Cellular immune responses of the insect Manduca sexta to bacteria and fungi

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CELLULAR IMMUNE RESPONSES OF THE INSECT MANDUCA SEXTA TO BACTERIA AND FUNGI

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Dedicated to my family

Dad, Mum, James, Mark, Karen

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Abstract

This thesis examines the cellular immune responses of the tobacco hornworm, *Manduca sexta* to bacterial and fungal infection.

Haemocyte monolayers were prepared from larvae that were previously injected with fluorescein-labelled heat-killed *Escherichia coli.* Most haemocytes in the monolayers from injected larvae were not involved in phagocytosis and the level of ingestion by the phagocytic haemocytes was low (1-3 bacteria per cell). Within the monolayer, however, was a low but consistent proportion of haemocytes that displayed many properties of 'professional phagocytes' as they phagocytosed bacteria to an extreme degree. These hyperphagocytic (HP) cell types have not been described previously in any invertebrate.

HP cells were rare in the haemocyte population (about 2% of total haemocytes) but were always present and constituted the main phagocytic response to the injected bacteria. These cells were also present in healthy, non-injected insects and were characterised by an extensive spreading ability and stellate morphology *in vitro* in the absence of bacteria. When incubated with bacteria, these well-spread cells engulfed an enormous number of bacteria revealing themselves to be HP cells. Evidence is presented that suggest HP cells play an important role in the initiation of nodule formation.

Eicosanoids are known to have a role in nodule formation during bacterial infection. The present work establishes that nodule formation is important in the defence against the fungus *Metarhizium anisopliae* and that this immune response is suppressed in the presence of eicosanoid biosynthesis inhibitors. These inhibitors had a direct suppressive

effect on the ability of haemocytes to form nodules *in vitro*. This work suggests that eicosanoids are important in regulating nodule formation during fungal infection.

M. sexta haemocytes responded to infection with *Beauveria bassiana* by a dramatic increase in spreading ability. Haemocyte spreading on glass coverslips was extreme during early infection but declined as the infection progressed. This spreading response was induced following infection with another fungus (*M. anisopliae*), pathogenic and non-pathogenic bacteria and laminarin. Evidence is provided that the hyperspreading haemocytes are also involved in nodule formation.

Both nodule formation and phagocytosis were strongly suppressed by culture supernatants of the entomopathogenic bacterium *Photorhabdus luminescens*. In addition, dead *P. luminescens* cells were phagocytosed and nodulated at much higher levels than live bacteria suggesting this pathogen actively suppresses these cellular immune responses. The possible factors and mechanisms that caused the immune suppression are discussed.

Abbreviations

χ^2	chi squared
° C	degrees celsius
AA	arachidonic acid
BSA	bovine serum albumin
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ENF	glutamic acid-asparagine-phenylalanine
FITC	fluorescein isothiocyanate
g	acceleration due to gravity
g	grams
GIM	Grace's insect medium
GR	granular cell
h	hours
HP	hyperphagocytic
kDa	kilodaltons
l	litre
LPS	lipopolysaccharide
μ	micro
m	milli
M	molar
mAb	monoclonal antibody
min	minutes
mm	millimetres
mosmol/kg	measure of osmolality
nm	nanometres
P	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH	measure of acidity
PL	plasmatocyte
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SDAY	Sabouraud's dextrose agar with yeast extract
SDS	sodium dodecyl sulphate
SE	standard error
sec	seconds
SEM	scanning elecron microscopy
TRITC	tetramethylrhodamineisothiocyanate
V	volts
VLBC	very large blood cells
v/v	volume for volume
w/v	weight for volume
YPD	yeast potato dextrose

Chapter 1

Introduction

Insects form symbiotic relationships with an enormous number of microorganisms. However, in most insect-microbe interactions, disease is the exception rather than the rule as the ability to invade, colonise and kill an insect is one that has been acquired by relatively few microorganisms.

The term 'entomopathogen' is collectively given to microorganisms that cause insect diseases. These include bacterial, fungal, nematode, protozoan and viral pathogens (Tanada and Kaya, 1992). For economic reasons, the field of insect pathology has mainly focussed on pathogens that cause disease in domesticated insects or insect pests. These insect 'models' have provided most of our knowledge about insect-pathogen interactions.

Despite recent advances in pest control, insects continue to represent globally the most serious competition for our food and, as vectors of parasites and pathogens, the greatest threat to our health (St. Leger and Bidochka, 1996). Thus, there is intense interest in the control of insect pests. Concerns over the environmental impact of synthetic chemical insecticides and the development insecticide resistance have resulted in an interest in biological alternatives including the use of entomopathogens. Optimisation of the employment of entomopathogens for biological control requires an understanding of the mechanisms of pathogenesis and the mechanisms of insect defence. There is intense interest in these two research areas and this introductory section of the thesis gives an overview of bacterial and fungal pathogenesis and the various defensive responses of the insect.

1.1 Entomopathogenic fungi

Fungi that cause disease in insects have been studied for over 150 years, since the seminal work of Bassi (1835), and at present over one thousand different species of entomopathogenic fungi have been identified (St. Leger and Roberts, 1997). Most species belong to the class Hyphomycetes, which are characterised by asexual reproduction and the production of a non-motile spore or conidium, which acts as the infective propagule.

The Hyphomycetes have the widest host range among entomopathogens with a single species usually consisting of many different isolates with a different host spectrum. Most of the current knowledge concerning fungal pathogenesis has been derived from the studies of two Hyphomycete species, *Metarhizium anisopliae* and *Beauveria bassiana.* These two species have wide host ranges with at least 200 insect species susceptible to *M. anisopliae* and nearly 500 species susceptible to *B. bassiana* (Vilcinskas and Gotz, 1999).

There are numerous reviews on the infection process of entomopathogenic fungi (Boucias and Pendland, 1998; Charnley, 1997; Clarkson and Charnley, 1996; Hajek and St. Leger, 1994; St. Leger, 1991). Most fungal pathogens enter the insect by direct penetration of the integument (the cuticle and epidermis) and rarely invade the host through the gut after being ingested. Fungal infections or 'mycoses' begin when contact is made between the cuticle of a susceptible host and a conidium. Conidia adhere to the cuticle, germinate, and produce a germ tube that elongates on the cuticle surface. Conidia usually have a hydrophobic surface that facilitates their attachment to the surface of the cuticle (Boucias and Pendland, 1991). After a short phase of fungal growth, penetration of the cuticle usually begins with the formation of a hyphal swelling known as the appressorium. This structure adheres strongly to the cuticle surface and is the focal point for enzymatic and mechanical penetration of the cuticle.

These pre-penetration events are usually highly sensitive to the environmental conditions and host cues. High humidity (usually over 90% relative humidity) and temperature are often very important factors for conidial germination. In addition, host factors such as cuticle hardness and topography often determine the success of the penetration process.

The insect cuticle is a highly effective barrier to infection (reviewed by St. Leger, 1991). Fungi are the only microbial entomopathogens that can infect actively by this route and bacteria and viruses often rely on wounds or enter via the gut. The importance of the cuticle in defence is revealed upon wounding, which allows normally innocuous microorganisms to gain entrance to the haemocoel, and often results in speedy insect mortality (Madelin, 1968, cited by St. Leger, 1991).

The fungal penetration process has been studied in some detail by St. Leger and co-workers (St. Leger, 1991). From the appressorium, a penetrant hypha emerges and grows

through the cuticle. As the insect cuticle is a complex biochemical structure, composed of proteins, lipids and chitin, entomopathogenic fungi usually possess a battery of hydrolytic enzymes to aid penetration and to also to provide nutrition for the fungus during penetration. Most research on cuticle degrading enzymes has concentrated on Pr1, a subtilisin-like protease produced by *Metarhizium anisopliae* (St. Leger *et al.*, 1987; St. Leger *et al.*, 1994). The importance of this enzyme as a virulence factor has been revealed using antibodies or specific protease inhibitors which cause a delay in mycosis development (St. Leger *et al.*, 1988). Also, Pr1-minus mutants of *M. anisopliae* show reduced virulence against *M. sexta* larvae (St. Leger, 1995), whilst genetically engineering strains of Pr1-overexpressing mutants was shown to enhance the fungal speed of kill of the insect (St. Leger *et al.*, 1996).

Upon entry into the haemocoel (usually between 24-48 hours post-infection), most entomopathogenic fungi differentiate and form yeast-like hyphal bodies or blastospores. *Beauveria bassiana* sheds its cell wall *in vivo* and disseminates within the haemolymph by way of protoplasts (Pendland *et al.*, 1993). This strategy may enable it to overcome the immune system because the protoplasts do not appear to be recognised by circulating *Spodoptera exigua* haemocytes (Pendland *et al.*, 1993). *B. bassiana* is also known to produce factors that suppress the immune response of *S. exigua*, suggesting that this pathogen uses multiple strategies to overcome the immune system (Boucias *et al.*, 1995).

Within the haemolymph, fungal pathogens produce a wide variety of metabolites including factors that suppress the immune system (Boucias *et al.*, 1995) and insecticidal toxins that kill the insect (Kershaw *et al.*, 1999). Several fungal-derived enzymes are secreted into the haemolymph during infection including proteases (Shimizu *et al.*, 1993), trehalases (Clarkson *et al.*, 1998), glucosidases (Clarkson *et al.*, 1998) and acid phosphatases (Xia *et al.*, 2000). These enzymes are thought to aid in nutrition and immune suppression and may also contribute to host tissue damage.

Host death is often due to a combination of factors including the actions of fungal toxins, tissue damage, autointoxication, nutrient depletion and obstruction of haemolymph circulation. Following death, fungal growth continues saprophytically and leads to the development of mycelium. Hyphae emerge from the cadaver and, under suitable conditions, sporulation occurs on the surface. Conidia are then released into the

environment until a susceptible host cuticle is encountered and the infection cycle continues.

1.1.2 Fungal metabolites

M. ansiopliae and *B. bassiana* are highly virulent insect pathogens that produce toxic metabolites *in vivo* and *in vitro*.

Both *M. anisopliae* and *B. bassiana* are facultative pathogens and can be grown away from the host in culture medium. Several biocidal secondary metabolites of entomopathogenic fungi have been detected in culture filtrates, but for most of these compounds, their role in pathogenesis is unclear. This is true for beavericin and bassianolide, toxic cyclic peptides produced *in vitro* by *B. bassiana* that have insecticidal properties (Champlin and Grula, 1979; Vey *et al.*, 2001). Several classes of enzymes are also produced *in vitro* by *B. bassiana* and *M. anisopliae* and these are thought to have putative roles *in vivo* (St. Leger *et al.*, 1986). Griesh and Vilcinskas (1998) report that proteases released *in vitro* by *M. anisopliae* and *B. bassiana* inhibit the spreading and phagocytosis by insect haemocytes, two important immune mechanisms.

Many isolates of *M. anisopliae* produce insecticidal toxins called destruxins (for a recent review see Vey *et al.*, 2001). This family of small cyclic peptides cause insect death when injected into insects and lethal concentrations of destruxins have been isolated from *M. anisopliae*-infected insects (Suzuki *et al.*, 1971). In addition, the destruxins have been found in amounts *in vivo* that correlate with toxicosis and the differential virulence of *M. anisopliae* isolates (Kershaw *et al.*, 1999). Thus, destruxins are unique among the mycotoxins because they have been shown to be important in pathogenicity. Physiological effects of destruxins are varied and often depend on the insect species in question. Toxicity and symptoms are most acute in Lepidopteran larvae and adult Diptera. Effects include inhibition of haemocyte (Vey *et al.*, 1985) and Malphigian tubule function (James *et al.*, 1993), midgut cytopathological effects (Dumas *et al.*, 1996), muscle paralysis (Samuels *et al.*, 1988) and inhibition of ecdysone synthesis (Sloman and Reynolds, 1993). It is not known whether the varied effects of the destruxins are due to a common mode of action. Destruxin B has been shown to be an inhibitor of vacuolar-type

ATPase, which is a vital enzyme in cellular physiology and its inhibition could account for many of these observed effects of the destruxins (Muroi *et al.*, 1994).

Recently, work with *B. bassiana* has shown that this fungus produces a high molecular weight toxin (greater than 10 kDa) in infected larvae (*S. exigua*). When infected haemolymph was injected into naive larvae, the larvae melanised and died (Mazet *et al.*, 1994) and the purified toxic factor was found to inhibit haemocyte spreading (Mazet *et al.*, 1994).

1.2 Entomopathogenic bacteria

Bacteria that cause diseases in insect can be divided into two classes: the spore forming and non-spore forming. These will be considered separately, although the nematode associated non-spore forming bacteria will be described in most detail.

1.2.1 Non-spore forming bacteria

Most non-spore forming bacteria are generally opportunistic pathogens and are themselves not actively pathogenic. These bacteria usually require the insect to be compromised in some way such as a wound or nematode invasion. However, upon entry into the insect haemocoel, non-spore forming bacteria are usually extremely virulent and only a few bacterial cells are required to kill the insect (Lysenko, 1985). Once inside the haemocoel, non-spore forming bacteria produce various toxic enzymes including cytolytic enzymes such as phospholipase C (*P. fluorescens*), proteases (*P. aeruginosa*) and chitinases (*S. marcescens*).

Although most non-spore forming bacteria are truly opportunistic pathogens and lack mechanism to gain entry to the haemocoel (e.g. *S. marcescens* and *P. aeruginosa*) some are actively invasive. *S. entomophila,* the causative agent of amber disease in the grass grub *Costelytra zealandica,* is a true pathogen as it is actively invasive and able to penetrate the cuticular lining of the foregut (Jackson *et al.*, 1993). Interestingly, this pathogen possesses pathogenicity determinants that share much sequence similarity with

the Tc toxins of the non-spore forming bacterium *P. luminescens*, which are active towards the insect midgut (described below; Hurst *et al.*, 2000).

The nematode-associated bacteria fall into two genera (*Photorhabdus* and *Xenorhabdus*) and these will be described in some detail due to their importance in the context of this project. Members of the genus *Photorhabdus* and *Xenorhabdus* are mutualistically associated with soil-inhabiting entomogenous nematodes (Forst and Clarke, 2001). *Xenorhabdus* and *Photorhabdus* species exist in the intestine of infective juveniles of nematodes belonging to the Steinernematidae and Heterorhabditidae families respectively. The nematodes enter the insect via natural openings or directly through the insect cuticle. Within the haemocoel, the nematodes release the bacteria from their gut and the bacteria multiply quickly and kill the insect usually within 48 h. *In vivo*, the nematodes are thought to feed on the bacteria within the dead insect, which grow exponentially to stationary phase and completely dominate the microbial flora. Eventually, the nematodes reassociate with the bacteria and develop into nonfeeding infectious juveniles, which leave the insect cadaver in search of new hosts. The infection process has been described in several reviews (Gaugler, 2001; Kaya and Gaugler, 1993).

Both *Photorhabdus* and *Xenorhabdus* are Gram-negative motile bacteria that are extremely virulent towards a wide range of insect larvae. The bacteria alone (without the nematode) are highly virulent when injected into larvae and work in recent years has started to unravel the mechanisms of their pathogenicity. Both *Xenorhabdus* and *Photorhabdus* species can be grown *in vitro* in nutrient medium where they secrete a wide variety of metabolites including lipases (Clarke and Dowds, 1995), proteases (Schmidt *et al.*, 1988), antibiotics (Forst and Nealson, 1996) and toxic compounds (Bowen *et al.*, 1998). These products have putative roles in infected insects; thus, the antibiotics prevent putrification of the cadaver by other microorganisms; the proteases and lipases enable the bioconversion of insect tissue into a usable nutrient form; and the toxins and other enzymes aid in immunosuppression and insect death.

P. luminescens, a bioluminescent bacterium (Schmidt *et al.*, 1989), produces a spectrum of metabolites *in vivo* and *in vitro*. Lipase produced by this pathogen is thought to be an important virulence factor. Spent culture medium of non-pathogenic *E. coli* carrying a plasmid encoding a *P. luminescens* lipase gene were insecticidal to *G. mellonella* (Clarke

and Dowds, 1995). Lipopolysaccharide (LPS) is also thought to be an important factor in the pathogenesis of *P. luminescens*. Like other Gram-negative bacteria, the outer membrane of *P. luminescens* contains LPS molecules, which comprise three parts: a lipid A anchor, a core polysaccharide and a hydrophilic O-antigen polysaccharide (Prescott *et al.*, 1996). LPS molecules are often highly active biological compounds in both vertebrates and invertebrates and can act as bacterial endotoxins responsible for many symptoms of bacterial infection (Prescott *et al.*, 1996). The LPS released by *P. luminescens in vivo* and *in vitro* has been shown to damage insect haemocytes and thus may be involved in immune suppression during infection (Dunphy, 1995).

High molecular weight insecticidal toxin complexes (Tc) that are active against a wide range of insects have been identified in spent culture supernatants of *P. luminescens* (Bowen *et al.*, 1998). Toxin complexes display oral activity when fed to larvae due to destruction of the midgut epithelium (Blackburn *et al.*, 1998; Bowen *et al.*, 1998; Silva *et al.*, 2002) and similar histopathological effects on the midgut occur following injection of the toxins (Bowen and Ensign, 1998). Four types of toxin complex have been identified (Tca, Tcb, Tcc Tcd), which vary in their oral activity. Gene knockouts of Tca and Tcd eliminate the oral activity of the culture supernatant suggesting these two toxin complexes are responsible for the oral activity. However, knockouts of Tcb and Tcc alone also reduced the associated mortality of the insects by the culture supernatant (Bowen *et al.*, 1998). The reason for the production of more than one Tc toxin is unclear but it is possible that each is active against different insect species (ffrench-Constant and Bowen, 1999) as the nematode symbiont infects a range of soil dwelling insect species. The Tc toxins may also be differentially active toward various target sites *in vivo* or may act together in a synergistic fashion *in vivo* to effect toxicity. Recently, the toxin complexes Tca and Tcd, which are highly orally active, were found to be produced by the bacteria within infected insects (Daborn *et al.*, 2001; Silva *et al.*, 2002). This finding, that the toxins are produced *in vivo* strengthens the hypothesis that these factors are important in the pathogenesis of *P. luminescens*. Interestingly, *P. luminescens* colonises the anterior midgut early in infection and here the bacteria, in close association with the midgut cells produce the Tc toxin Tca, which has high oral activity (Silva *et al.*, 2002).

Another putative virulence factor of *P. luminescens* includes the recently identified toxin mcf (makes caterpillars floppy). The mcf gene, when transferred to *E. coli*, confers to the

bacteria the ability to persist within *M. sexta* larvae and kill the insects (Daborn *et al.*, 2002). Mcf toxin, when expressed in *E. coli* results in a loss of insect body turgor followed by insect death. Under normal conditions, *E. coli* is non-pathogenic and rapidly cleared from the haemolymph by the immune system. The sequence of the mcf toxin shows little homology with known protein sequences but it does possess a BH3 domain, which is characteristic of pro-apoptosis proteins. The mcf toxin appears to cause apoptosis in *M. sexta* haemocytes and the insect midgut epithelium. Cytosolic fractions of *E. coli* carrying the mcf toxin also caused blebbing of granular cells and actin cytoskeletal disintegration of the haemocytes (Daborn *et al.*, 2002).

Given that *P. luminescens* replicates in the haemolymph with apparent disregard for immune responses, it follows that these bacteria must possess mechanisms for the avoidance or suppression of insect immunity. As few as 5 bacteria, when injected into larvae have been reported to kill the insect (Gotz *et al.*, 1981). As mentioned previously, the bacteria are known to secrete a variety of factors *in vitro* that are likely to have suppressive effects on insect haemocytes. Van Sambeek and Wiesner (1999) have shown that infection of desert locusts with *Heterorhabdus megidis* (containing *P. luminescens*), causes a strong suppression of the phagocytic competence of the haemocytes *in vivo* and also resulted in haemocyte death. The authors also report that cell-free culture supernatants of *P. luminescens* inhibit the locust phagocytes *in vitro*. The factors that enable the bacterium to overcome the immune system are at present unidentified. The toxin complexes themselves, although shown to have midgut actvity, may be active on insect haemocytes. Although the mode of action of Tc is unknown, they may act on vital cellular physiological processes, and therefore may affect many target tissues, including haemocytes. The mcf toxin also provides a mechanism for *P. luminescens* to suppress haemocyte-mediated responses.

It should be mentioned that similar virulence factors and mechanisms of pathogenesis have been reported for *Xenorhabdus* spp. There is much evidence to suggest that *Xenorhabdus* LPS is an important virulence factor. LPS released by *Xenorhabdus* has been shown to prevent the activation of phenoloxidase, an important enzyme in insect immunity and to damage insect haemocytes (Dunphy and Webster, 1988). Also purified LPS, when injected into *Galleria mellonella* larvae kill the insects demonstrating its toxicity (Dunphy and Webster, 1984).

In conclusion, *Photorhabdus* and *Xenorhabdus* species appear to possess a wide variety of virulence factors including toxic enzymes, numerous proteins with novel toxic properties and LPS. Genomic sample sequencing of *P. luminescens* (ffrench-Constant *et al.*, 2000) reveal this bacterium possesses genes that encode for many other putative virulence factors including haemolysins and may possess a type III secretion apparatus, a common virulence determinant employed by many bacterial pathogens. Type III secretion systems allows bacteria to inject proteins into host cells. It remains to be seen whether *P. luminescens* uses this strategy in infected insects upon interaction with insect cells.

1.2.2 Spore forming bacteria

This group of entomopathogenic bacteria are so named because they all share the ability to produce a highly resistant endospore. Although a number of spore forming bacterial species exist, the most extensively studied, due to its commercial applications, is *Bacillus thuringiensis* (Bt) and there are several reviews on the pathogenesis of this bacterium (Aronson and Shai, 2001; de Maagd *et al.*, 2001; Schnepf *et al.*, 1998). Bt is a Gram-positive insect pathogenic bacterium of massive agronomic and scientific interest. The use of Bt-based products for pest management provides a useful alternative to chemical insecticides and commercial production of Bt represents over 90% of the biological control market (Boucias and Pendland, 1998).

Sporulation of Bt occurs during the stationary phase of growth and results in the production of a dormant endospore. The endospore is highly resistant to environmental stresses and allows the bacterium to persist for long periods in the environment. During sporulation, Bt also produces large, highly toxic crystalline inclusions that contain insecticidal toxins called δ-endotoxins. Various forms of δ-endotoxins have been identified and referred to as Cry proteins, which have been classified into many different groups (Aronson and Shai, 2001). Different strains of Bt carry different types or different combinations of Cry proteins and this dictates their host specificity. As a species, Bt has a wide host range but individual strains are more specific, usually effective against species in the orders Lepidoptera, Diptera or Coleoptera. The individual Cry toxins have a

defined spectrum of insecticidal activity and have themselves been used as a biological insecticide in the form of sprays. Furthermore, the recent cloning of Cry protein genes and their expression in transgenic plants has provided an effective means for crop protection against insect pests (de Maadg *et al.*, 1999).

After Bt enters the insect orally after being ingested, the Cry proteins are solubilized and processed within the midgut environment (high pH and midgut proteolytic enzymes). The active toxins exert their activity on the midgut, where they form pores in membrane of the midgut columnar cells, disrupting the transmembrane potential and leading to osmotic lysis. The mode of action of Cry toxins have been recently reviewed by Aronson and Shai (2001). Intoxicated larvae quickly cease feeding and the paralysis of the gut ensures the bacterial endospores are not removed by peristalsis. As the gut wall is degraded, the pH of the gut lumen increases due to mixing with the haemolymph and this is thought to aid germination of the endospores. The insects usually die from either starvation or septicaemia.

Besides the Cry proteins, other virulence factors are also produced by Bt including cytolysins (Cyt toxins) found within the crystalline body, and factors secreted during vegetative growth like the vegetative insecticidal proteins (VIPs). Recently, Yu *et al.*, (1997) have shown that VIP3a causes gut paralysis when applied orally to black cutworm larvae and results in the death of this insect. VIP3a has been shown to be active on the midgut cells. This entero-toxic activity appears to be a common theme of effective toxins produced by entomopathogenic bacteria (cf. Tc toxins of *P. luminescens*). Transferring the gene encoding VIP3a to *E. coli* conferred insecticidal activity to these normally non-pathogenic bacteria (Estruch *et al.*, 1996).

It is apparent that Bt, like *P. luminescens*, possess a wide selection of virulence factors other than the δ-endotoxins including enterotoxins (Rivera *et al.*, 2000), VIPs (Estruch *et al.*, 1996), cytolytic toxins (Guerchicoff *et al.*, 2001) and α- and ß-exotoxins (Herbert *et al.*, 1985; Ohba *et al.*, 1981; Toledo *et al.*, 1999). However, the importance of these virulence factors has not been established.

Finally, it should be mentioned that with the wide spread use of Bt-based products, insect resistance to Bt has developed in some species (Lui *et al.*, 1999). Given that many biological control programs rely on Bt-based products, it is important to continue searching for novel biological insecticidal toxins. Toxins such as the VIP3a toxin of Bt, and the mcf and Tc toxins of *P. luminescens* provide potential alternatives to the Bt δ-endotoxins in insect control because all these toxins act on the insect midgut and may therefore be effective when ingested in the field.

1.3 The insect immune system

The insect immune system has been studied for over a century. It is the last line of defence for the insect against potential invaders after the cuticle has been breached. The immune system can be divided into two main components (1) cellular responses, which are carried out by the blood cells (haemocytes) and include phagocytosis, nodule formation and encapsulation (2) humoral (plasma) responses involving the production of antimicrobial peptides and proteins. Humoral immunity is an important component in insect immunity, however in the context of this project, this will only be considered briefly.

The insect immune system is often regarded to share many fundamental characteristics with the innate immune system of vertebrates (Vilmos and Kurucz, 1998). Despite its apparent simplicity when compared with vertebrate immunity, the insect immune system is complex and research in recent years has revealed the many endogenous factors that are involved. Genetic analysis with model organisms such as *Drosophila* is also providing a wealth of information on the underlying molecular mechanisms that regulate insect immune responses (de Gregorio *et al.*, 2001; Khush and Lemaitre, 2000) and has revealed many similarities with the mammalian innate immune responses (Hoffmann *et al.*, 1999).

This section describes the insect immune system with emphasis on cellular immune responses (phagocytosis and nodule formation). Several well-established reviews on insect and invertebrate immunity exist that provide an in-depth assessment of both cellular and humoral immune responses (Boman and Hultmark, 1987; Gillespie *et al.*,

1997; Gupta, 1991; Lackie, 1988; Millar and Ratcliffe, 1994; Ratcliffe and Rowley, 1979; Vilmos and Kurucz, 1998).

1.3.1 Insect haemocytes

Insects have an open circulatory system in which the haemolymph (blood) circulates freely in a body cavity or haemocoel. Movement of the haemolymph is maintained by contractions of a dorsal vessel, which move the haemolymph in an anterior to posterior direction. Insect haemolymph has many functions including transport of nutrients and hormones, removal of waste products, maintenance of a hydrostatic skeleton and mediation of immune responses.

The cellular immune responses (i.e. phagocytosis and nodule formation) are carried out by the insect blood cells (or haemocytes). The haemocytes float freely in the haemolymph or may sometimes be found as sessile fixed cells found attached to various tissues. There is much confusion and controversy in the classification of the haemocyte types and more than 70 different names of insect blood cells have been reported by different authors. It seems likely that the many different forms of insect haemocytes that are reported are variations of a few common cell types. The reasons for this inconsistency include variations in microscopical technique, variations in haemocyte morphologies between insect species and inconsistent *in vitro* culture conditions employed by different researchers. Indeed, as the number of insect species far exceeds that of any other group, it is not surprising that variations in blood cell types exist. A number of authors have put forward classification schemes for insect haemocytes including Price and Ratcliffe (1974) who have grouped insect haemocytes into 6 main classes from 15 insect Orders: prohaemocytes, plasmatocytes, granular cells, spherulocytes, oenocytoids and cystocytes. Gupta (1991) describes a similar scheme. This author also suggests that the term immunocytes should be used for the granular cell and plasmatocyte types because of their importance in the immune system. These two cell types have indeed been found by most authors to be the main effector cells in the insect immune system and they will be considered here in most detail. The other haemocyte types do not appear to have a major role in immune responses and are usually less common in the haemolymph than the plasmatocytes and granular cells.

The prohaemocytes are often thought to be the haemocyte stem-cells from which other haemocytes are derived. They are often very small and are rare in the haemocyte population (usually less than 5%). Yamashita and Iwabuchi (2001) show that in *Bombyx mori*, prohaemocytes differentiate into plasmatocytes or granular cells *in vitro*, supporting the view that prohaemocytes have a stem cell function.

The plasmatocytes (PL) are usually spindle shaped *in vivo* but upon attachment to glass slides, they characteristically spread by the formation of filopodial extensions. In general, the PL usually account for between 30-60% of circulating haemocytes in most insects (Rowley and Ratcliffe, 1981) and their functions vary but are commonly associated with phagocytosis and nodule formation. Interestingly, there is much variation in function of PL between species as in *Bombyx mori* (Wago, 1991) the granular cells are the main phagocytic cell type whereas in *Galleria mellonella* the PL are the principle phagocytes (Ratcliffe and Rowley, 1974). This exemplifies the difficulty with classifying haemocytes based on function alone and reveals the importance of both ultrastructural and functional studies.

The granular cells (GR) are commonly round or oval cells and unlike PL, do not spread extensively *in vitro*. The GR commonly degranulate *in vitro*, releasing their contents and leaving clear vacuoles in the cytoplasm. Thus, GR appear to be very sensitive to foreign material. Like PL, the GR have many different reported functions including phagocytosis, nodule formation, encapsulation and coagulation.

Numerous studies have characterised haemocyte types using their differential affinity for monoclonal antibodies or lectins (McKenzie and Preston, 1992b). This is extremely useful as it enables a more objective classification of the cell types (i.e. based on biochemical markers). The use of monoclonal antibodies to classify blood cells in vertebrates is well established and this technique has recently been used by insect immunologists to define different haemocyte types (Chain *et al.*, 1992; Gardiner and Strand, 1999; Mullet *et al.*, 1993; Scapigliati *et al.*, 1996; Willott *et al.*, 1994). Interestingly, many of the haemocyte ligands that are recognised by the antibodies have also been shown to be important in immune functions as antibody binding blocked haemocyte spreading (Wiegand *et al.*, 2000) and suppressed the formation of nodules (Mullet *et al.*, 1993).

Changes in the haemocyte profile of the haemolymph and the state and behaviour of insect haemocytes are caused by a variety of factors. These have been reviewed by Lackie (1988). Factors that affect the haemocyte population include the developmental status of the insect, stress and wounding and the presence of nonself within the haemocoel. In most cases following injection of foreign particulates, the haemocyte count decreases dramatically and this is thought to be partially due to the haemocytes being used up in events such as nodule formation. In particular, many studies have found that there is a strong depletion of the PL haemocyte class following bacterial injection (Chain and Anderson, 1982; Geng and Dunn, 1989). A plasmatocyte depletion factor in the haemolymph has been implicated that may cause a change in the adhesive properties of the PL (Chain and Anderson, 1983). Recently, plasmatocyte spreading peptides have been isolated from *M. sexta* (Wang *et al.*, 2001) and *Pseudoplusia includens* (Clark *et al.*, 1997), which both share high sequence homology (Strand *et al.*, 2000) and cause depletion of plasmatocytes following injection into respective larvae. Thus, these spreading peptides are likely candidates for the increased adhesive properties of PL during infection.

1.3.2 Recognition of foreignness and the prophenoloxidase cascade

Recognition of nonself is a prerequisite of immune systems. However, the mechanisms involved are probably the least understood aspect of invertebrate immunity.

The mechanisms of nonself recognition in invertebrates have been studied in detail but the process is still relatively fragmented compared to vertebrates. Although recognition of nonself may in part, be determined by physical properties such as surface charge or hyrdophobicity of the foreign particle (Da Silva *et al.*, 2000; Lavine and Strand, 2001), other more specific mechanisms of recognition mechanisms occur. Lackie (1988) provides a useful review on the mechanisms of recognition of nonself by insects haemocytes.

Invertebrates lack antibody-mediated specificity thus it is thought that the immune system does not recognise specific microbial epitopes but rather relies on receptors that recognise commonly conserved microbial structures or molecular 'patterns' (Janeway,

1992). It is clear that invertebrates have the ability to recognise specific microbial patterns such as lipopolysaccharide (LPS) and peptidoglycan from bacteria, and ß-1,3-glucans and mannans from fungal cell walls. Indeed, these microbial molecules are good elicitors of insect immune responses. Microbial patterns are highly conserved elements that are unique to the surface of many microorganisms and allow a broad spectrum of potential invaders to be detected. The *Drosophila* genome encodes a large number of proteins with putative recognition properties (Khush and Lemaitre, 2000), although to date there is no genetic demonstration for a role of these proteins in immunity (Tzou et al., 2002).

Many insect haemolymph proteins have been identified that specifically recognise and bind to these microbial molecular patterns and can collectively be termed pattern recognition receptors (Medzhitov and Janeway, 2000). These include among many, the Gram-negative binding protein (GNBP; Lee *et al.*, 1996), peptidoglycan recognition proteins (PGRP; Ochiai and Ashida, 1999; Werner et al., 2000) and ß-1,3-glucans binding proteins (Ochiai and Ashida, 2000). Lectins (carbohydrate binding proteins) constitute the main group of pattern recognition molecules and there are many examples of insect lectins in the literature.

Lectins are commonly synthesised in the fat body tissue (and sometimes in the haemocytes) and then released into the haemolymph. Some studies show that the synthesis of lectins is induced following infection or wounding (Komano *et al.*, 1980; McKenzie and Preston, 1992a). Pattern recognition molecules such as lectins are often reported to serve as opsonins, allowing enhanced recognition of microorganisms by the haemocytes. Lectins act as bridging molecules between the haemocyte and the microorganism surface as they are thought to possess more than one binding site. This has been demonstrated with hemolin, which binds to bacteria and haemocytes. Hemolin, is a 48-kDa protein synthesized by haemocytes and fat body in *M. sexta* and *Hyalophora cecropia* (Ladendorff and Kanost, 1991; Sun *et al.*, 1990). This protein is induced to high levels in the haemolymph upon injection of bacteria or bacterial LPS and has been shown to bind to the bacterial cell surface and the haemocyte membrane and stimulate the phagocytic uptake of the bacteria (Zhao and Kanost, 1996). Although only a few opsonins have been identified, the plasma of many insects has been shown to display opsonic properties.

Another source of opsonins are the components of the prophenoloxidase (PPO) cascade. PPO is an inactive enzyme that is activated to phenoloxidase (PO) during immunological challenge and results in the production of melanin. PPO exists in the plasma and in the insect cuticle and the formation of melanin at these two sites is a common response to infection suggesting this enzyme is an important component in insect defence. The activation of PPO has been studied in some detail, particularly in crustaceans (reviewed by Soderhall and Cerenius, 1998). Many microbial elicitors have been found to activate the PPO cascade including whole bacteria and fungi and microbial cell wall components such as ß-1,3-glucan and LPS (Soderhall and Hall, 1984). In addition endogenous recognition proteins, upon binding to microbial molecular pattern have been reported to stimulate the activation of PPO (Ma and Kanost, 2000).

Although the process of PPO activation is complex, the process can be summarised by (1) recognition of nonself by plasma or haemocyte receptors leads to the activation of serine proteases (2) the serine proteases convert the PPO to an active form (3) PPO activation is associated with the production/activation of numerous other proteins including some that adhere to the surface of foreign particles and act as opsonins (Soderhall *et al.*, 1984). Also LPS and ß-1,3-glucans, which are known activators of PPO cascade have both been shown to enhance phagocytosis and nodule formation *in vitro* implying an opsonic role of PPO components.

To conclude this section on nonself recognition, research on the fruit fly, *D. melanogaster* has revealed that the immune system can discriminate between different microorganisms. Bacteria and fungi, when injected into this insect activate different signal transduction pathways and cause the expression of different immune-related genes (Lemaitre *et al.*, 1997); fungi stimulate the specific synthesis of antifungal proteins while bacteria stimulate the synthesis of antibacterial proteins. Gram-negative and Gram-positive bacteria also induce different patterns of immune gene expression (for a recent review see Khush and Lemaitre, 2000). This implies that insects possess specific recognition molecules and specific signal transduction pathways depending on the type of nonself that is present. One of the recognition receptors involved in the signal transduction pathway has recently been identified as a peptidoglycan recognition protein (Michel *et al.*, 2001), a common recognition receptor in insects (Dimopoulos *et al.*, 2000; Ochiai and Ashida, 1999; Werner *et al.*, 2000).

1.3.3 Humoral immunity

The humoral immune response concerns factors that are present in the plasma that contribute to blood-borne defence such as antimicrobial peptides/proteins and serum lectins. Although lectins are important humoral factors, these have been considered previously. Following immune challenge, antimicrobial peptides and proteins are rapidly and transiently synthesised by the fat body, although synthesis by haemocytes and epidermis has been reported.

Pioneering work on humoral immune responses was performed by Hans Boman and co-workers on the pupae of the cecropia moth, *Hyalophora cecropia* (Boman and Hultmark, 1987; Steiner *et al.*, 1981). These authors reported that injection of bacteria into diapausing pupae resulted in the synthesis of immune proteins in the haemolymph, most notably the antimicrobial peptides cecropin, attacin and lysozyme. It should be pointed out that the cellular immune responses such as phagocytosis and nodule formation (discussed below) are responsible for the rapid removal of bacteria from circulation (usually within the first 2 h following injection of bacteria) and that humoral immunity is a more developed and longer lasting response (i.e. days). In *H. cecropia* pupae, the humoral proteins peaked on day 7 and 8 following injection, after which the activity gradually declined. Cecropins were the main antibacterial proteins in the challenged pupae. Similar humoral responses occur in *M. sexta* as bacterial injection induces the synthesis of many proteins, with the main proteins having sequence similarity to the cecropins, attacins and lysozyme.

The different antimicrobial proteins synthesised following immune challenge act synergistically by attacking different target sites of the bacteria; attacins affect cell division of Gram-negative bacteria; lysozyme breaks down peptidoglycan found in both Gram-positive and Gram-negative bacteria; cecropins act on the lipid membrane in Gram-positive and Gram-negative bacteria; defensins (a widespread and more recently discovered class of antimicrobial peptides) act on the membranes of Gram-positive bacteria.

Many humoral immune proteins, from many different insect species have now been isolated and the number exceeds 150 different proteins (Hoffmann and Reichhart, 1997).

One recently discovered protein is drosomycin, an antifungal peptide induced by bacteria injection in *D. melanogaster* (Fehlbaum *et al.*, 1994). Interestingly, drosomycin was found to be synthesised in epithelial tissues suggesting a localised humoral response to fungi occurs in the epithelium as the fungus breaches the cuticle (Ferrandon *et al.*, 1998).

The induction of humoral immune proteins by microbial infection is not a ubiquitous phenomenon of insect immunity. Some species of cockroach show no humoral induction to infection and instead appear to have a very efficient cellular immune system and huge titres of lectins within the plasma. For most insects that have been studied, however, the response to bacterial infection results in the synthesis of many antibacterial peptides and proteins that are released into the haemolymph. These proteins provide a backup to the cellular immune responses and also provide protection against further bacterial infections, which would be common when the insect is wounded.

1.3.4 Cellular immune responses

Haemocytes are able to distinguish between self and nonself. It is generally accepted that upon recognition of nonself, the main response of the haemocytes is to spread (Gillespie *et al.*, 1997). If the foreign agent is small then the spreading response results in the phagocytosis of the particle, whereas larger foreign objects (or many small foreign particles) would be encapsulated (or nodulated). Spreading may also help to seal wounds. Thus, the ability to spread on glass coverslips *in vitro* is a common property of blood cells and this parameter is commonly assessed as a measure of immune fitness.

The fact that most haemocytes are round or ovoid in their native state *in vivo* demonstrates the strong reaction that these cells have to the glass coverslip. After a few minutes exposure to the glass surface, the response of the haemocytes is to spread, suggesting the haemocyte recognises the surface as foreign. Some authors believe the spreading response of blood cells is a 'frustrated phagocytosis' in that these cells are attempting to phagocytose the glass slide (Swanson and Baer, 1995). In insects, another possibility is that the haemocytes are attempting to encapsulate the slide (see below).

Haemocytes participate in three main cellular immune responses: phagocytosis, nodule formation and encapsulation, and these will be described separately.

1.3.4.1 Phagocytosis

Phagocytosis is a ubiquitous mechanism in both vertebrates and invertebrates and can be considered as the ultimate process for the disposal of foreign particles by the host. Phagocytosis in its simplest terms is the uptake of relatively large particles by the cell. The process has been extensively studied in vertebrates and much of the underlying cell biology has been elucidated (Brown, 1995; Greenberg, 1995) but the process in insects is not well understood.

Phagocytosis is a complex cellular process but can be divided into three main stages. (1) attachment of the phagocyte to the foreign particle (2) ingestion (3) digestion and/or killing of the particle. Recognition of the particle by insect haemocytes is similar to that described above and is likely to rely on the specific interaction between the foreign particle and cell surface receptors or indirectly by bridging molecules. Bridging agents that enhance phagocytosis are commonly referred to as opsonins and include lectins (Pendland and Boucias, 1996) and components of the prophenoloxidase system (Ratcliffe *et al.*, 1984). Haemolymph has been shown to contain opsonins and the inclusion of plasma in phagocytosis assays *in vitro* has been found to stimulate phagocytic activity (Rohloff *et al.*, 1984). Interestingly, factors released by GR in *G. mellonella* specifically enhance phagocytosis by the PL (Anggraeni and Ratcliffe, 1991) and ß-1,3-glucans was important in stimulating the release of these opsonisation factors by GR. Wiesner and Gotz (1993) also show that phagocytic recognition in *G. mellonella* is dependent on the physical characteristics of the particle surface, as hydrophobic beads do not provoke a strong response whereas hydrophilic beads are phagocytosed. In another study, *G. mellonella* haemocytes show very high phagocytic activity (i.e. 86% of haemocytes) towards non-opsonised glass beads (Ehlers *et al.*, 1992). As beads do not possess microbial molecular patterns and were not opsonised in these experiments, the importance of opsonisation and indeed receptor mediated phagocytosis in insects is unclear.

Following recognition, the foreign particle is ingested. This occurs in most cases by the invagination of the plasma membrane, which surrounds the particle and envelopes it within a phagocytic vacuole or phagosome. Thus, for each particle engulfed, there is a loss in plasma membrane and this is likely to be a limiting factor for high phagocytic

activity. It has been demonstrated in vertebrate phagocytes that optimal phagocytosis only occurs concomitantly with exocytosis, which allows the plasma membrane to be replenished (Hackam *et al.*, 1998).

Killing in insects of intracellular microbes within the phagosome is thought to occur by the action of toxic oxygen metabolites, lysosomal enzymes including lysozyme, toxic quinones, antimicrobial peptides and low pH (Millar and Ratcliffe, 1994). However, although killing mechanisms in vertebrate blood cells are well established (Underhill and Ozinsky, 2002), and bacteria killing clearly occurs in insect phagocytes, relatively little is known about the killing mechanisms by insect phagocytes.

Some pathogens, when phagocytosed, can tolerate the intracellular environment of the phagocyte and may use haemocytes as a haven to develop and multiply. This is a very common strategy of many mammalian pathogens (Amer and Swanson, 2002). *M. anisopliae* has been shown to use this strategy as hyphal bodies of this fungus are not killed within PL of *G. mellonella*, but propagate and grow within individual haemocytes (Vilcinskas and Gotz, 1999). Similar findings have been found with *B. bassiana* (Boucias and Pendland, 1998).

The main phagocytic haemocyte types in insects are the PL and GR, although their relative contribution differs among insect species; in *M. sexta* both GR and PL are phagocytic; in *B. mori* only the GR is phagocytic (Wago, 1991); in *Galleria mellonella*, the PL are the main phagocyte type (Ratcliffe and Rowley, 1974). Fixed phagocytes have also been reported in the haemocoel of a number of invertebrate species (Johnson, 1987; Matricon-Gondran and Letocart, 1999; Sminia *et al.*, 1979) and phagocytic organs have been found in the haemocoel of crustacea (Cuénot, 1905) and insects (Hoffmann *et al.*, 1974; Jones, 1970). These cells typically exhibit a high phagocytic activity toward injected particles, often to exclusion of the circulating haemocytes (Reade, 1968).

In insects, phagocytosis by haemocytes occurs against both fungi (Hung *et al.*, 1993; Vilcinskas and Gotz, 1999) and bacteria (Wilson and Ratcliffe, 2000) and has been shown to be an important immune reaction during infection (Horohov and Dunn, 1983). Phagocytosis has been commonly linked with the clearance of low doses of bacteria. Ratcliffe and Walters (1983) quantified this phenomenon in *G. mellonella* and report that

doses of less than 1000 bacteria per microlitre are removed by phagocytosis whereas larger doses are principally removed by nodule formation. In *M. sexta*, Horohov and Dunn (1983) report that bacteria are cleared by nodule formation during the first 2 hours after injection, and thereafter (2-8 hours) are removed by phagocytosis.

In the past, measuring phagocytosis was performed by light microscopy alone. This technique has many limitations and is often subject to the interpretation of the user as it is difficult to assess whether particles are attached to the phagocyte or whether they have been ingested. Transmission electron microscopy can get around this problem but is often very tedious and time consuming. Many methods are now available to assess phagocytosis quickly and the most popular is fluorescent quenching (Hed, 1977; Rohloff *et al.*, 1994). Briefly, this involves labelling foreign particles with a fluorochrome, which is quenched by agents such as trypan blue. As trypan blue is a cell-impermeable dye, it cannot quench intracellular (phagocytosed) particles and these retain their fluorescence. Other common techniques that are used to quantify phagocytosis include confocal microscopy (Babcock, 1999; Hook and Odeyale, 1989), immunofluorescence (Goosney *et al.*, 1999) and flow cytometry (Ramarao and Meyer, 2001).

Many endogenous and exogenous factors are known to influence phagocytosis. As mentioned previously, ß-1,3-glucans (Gunnarson, 1988) and LPS (Wittwer *et al.*, 1997) enhance phagocytosis and this is probably due to the release of opsonins such as components of the PPO cascade from the haemocytes. A number of identified endogenous factors that affect phagocytosis have recently been reported. These include lectins (Wilson *et al.*, 1999), hormones such as octopamine (Baines *et al.*, 1992), apolipophorin III (Wiesner *et al.*, 1997), the eicosanoids (Mandato *et al.*, 1997), and uncharacterised phagocytosis-stimulating factors (Rohloff *et al.*, 1994; Wiesner *et al.*, 1996). Phagocytosis suppressive factors have also been identified from bacterial (Silva *et al.*, 2002; included in the present study) and fungal pathogens (Vilcinskas *et al.*, 1997a).

1.3.4.2 Nodule formation and encapsulation

Nodule formation is a multicellular immune response in which many haemocytes surround and trap large doses of foreign particulate material in a cellular aggregate or nodule. Nodule formation is thought to occur when the particulates cannot be effectively

cleared by phagocytosis alone. Many foreign elicitors have been reported to stimulate the formation of nodules including fungi (Bidochka and Khachatorians, 1987; Gunnarson and Lackie, 1985; Vey and Fargues, 1977), bacteria (Horohov and Dunn, 1982; Miller *et al.*, 1994), ß-1,3-glucans (Gunnarson and Lackie, 1985) and bacterial LPS (Ratcliffe *et al.*, 1991).

Nodule formation and encapsulation are essentially very similar immune responses against microorganisms or larger foreign objects respectively. Insect endoparasitoid wasps, which inject their eggs into insect hosts, and invading nematodes, which penetrate into the haemocoel, are usually the natural target for encapsulation while fungi and bacteria are the common elicitors of nodule formation. Encapsulation has been reviewed previously by various authors (e.g. Davies and Siva-Jothy, 1991).

The sequence of events of nodule formation has been described in detail in many reviews (Ratcliffe and Rowley, 1979; Rowley and Ratcliffe, 1981) and the description given is adapted from the biphasic model of nodule formation outlined by Rowley and Ratcliffe (1981) in *G. mellonella*. Firstly, following the injection of bacteria, GR quickly attach to the bacteria and degranulate (release the contents of intracellular vacuoles) resulting in a flocculent material deposited at the site of attachment. GR are typically sensitive to foreign material in *G. mellonella* and frequently disintegrate *in vitro*. A clot forms and this entraps the bacteria. Second, large numbers of PL attach to the central melanising and necrotic core of the growing nodule (1-6 hours post-injection). These PL, which may contain phagocytosed bacteria, rapidly flatten down to form a series of layers around the growing nodule. It is believed that the PL are attracted to the nodule through chemotactic factors as *in vitro* experiments show unidirectional movement of PL toward nodules (Lackie, 1988). Large mature nodules are finally removed from circulation by attachment to the body wall (Ratcliffe and Gagen, 1976).

Nodule formation is an extremely rapid immune response and occurs within minutes of particulate injection. Its effectiveness against bacterial infections has been shown in *M. sexta* by Stanley-Samuelson *et al.* (1991) and Miller *et al.* (1994) in which inhibition of nodule formation by eicosanoid biosynthetic inhibitors enhanced bacterial septicaemia and death of this insect. Earlier studies with *M. sexta* found nodule formation was responsible for the clearance of 90% of injected bacteria within the first 30 min (Horohov

and Dunn, 1983). Although phagocytosis and nodule formation were reported to occur concomitantly in *M. sexta*, phagocytosis of the bacteria was only significant between 2-8 hours post injection (Horohov and Dunn, 1983).

Within the nodule, the bacteria quickly lose viability. Bacterial death may be the results of toxic metabolites released by the haemocytes or physical stresses such as lack of oxygen. Interestingly, *Bacillus cereus*, a bacterial pathogen of *G. mellonella* elicits a nodule response but does not succumb to the killing mechanisms within the nodule. This pathogen multiplies within the nodule and escapes into the haemolymph (Walters and Ratcliffe, 1983). This occurs with fungal pathogens such as *M. anisopliae* (Vilcinskas and Gotz, 1999) and *B. bassiana* (Bidochka and Khachatorians, 1987) both of which have been reported to grow out of the nodules.

Foreign particles that are too large to be phagocytosed are often encapsulated. The sequence of events during capsule formation is similar to that described for nodule formation. Thus, GR are assumed to contact the foreign object randomly and release chemotactic components that attract PL which form multicellular layers around the object. Spreading of PL is an important step in the formation of a capsule and this may explain why PL spread extensively on glass slides *in vitro*. Although GR are thought to be the initiators of the encapsulation response, Gillespie *et al.* (1997) report that in *M. sexta*, the PL are important in the early stages of encapsulation *in vitro* thus the cell types involved may be quite different between species. In certain Diptera, the process of encapsulation is very different, as haemocytes are not involved. In these insects, the haemocyte count is low and humoral or melanotic encapsulation occurs in which phenoloxidase activity results in the deposition of melanin on the surface of invading organisms. In *D. melanogaster*, encapsulation is associated with a specialised cell type that is induced upon wasp parasitisation or implantation of a large foreign object in the haemocoel. These new cells or lamellocytes are large flat cells that are released into the haemolymph following immune challenge and form layers around the foreign object.

1.3.4.3 Modulators of encapsulation and nodule formation

Nodule formation and encapsulation are complex events. Endogenous factors that mediate these responses are beginning to be identified. Although many endogenous

factors that affect nodule formation have been reported, the most extensively studied are the eicosanoids. Eicosanoids are lipid signalling molecules with highly potent biological activity that have been well characterised in mammals because of their role in inflammation. It is now recognised that eicosanoids mediate nodulation during bacterial infection in insects, a hypothesis now formalised by Stanley (2000). Although all of the work in this area of research relies on the use of eicosanoid biosynthetic inhibitors that are typically used in mammals, work has shown that haemocytes (Gadelhak *et al.*, 1995) and fat body tissue (Stanley-Samuelson and Ogg, 1994) in insects synthesise eicosanoids and that this is inhibited in the presence of the eicosanoid biosynthetic inhibitors. Furthermore, the suppressive effects of these inhibitors on nodule formation have been found to be reversed in the presence of arachidonic acid, an eicosanoid precursor. Jurenka *et al.* (1999) also show that the eicosanoid prostaglandin is stimulated in the insect *Pseudaletia includens* by bacterial infection and that this production is inhibited with the pharmacological inhibitors. Since the work of Miller *et al.* (1994) with *M. sexta*, other workers have found eicosanoid biosynthetic inhibitors to suppress nodule formation to bacteria in many other insects (Jurenka *et al.*, 1997; Miller *et al.*, 1999; Stanley *et al.*, 1999; Tunaz *et al.*, 1999). Moreover, the role of eicosanoids in nodule formation does not appear to be restricted to bacterial infections as recent work has shown that eicosanoids influence the nodule response to LPS (Bedick *et al.*, 2000) and latex microspheres (Mandato *et al.*, 1997). Most recently Carton *et al.*, (2002) have shown encapsulation of parasitoid eggs to be suppressed in the presence of the eicosanoid biosynthetic inhibitors.

Other modulators of nodule formation and encapsulation include haemocyte adhesion molecules such peptides containing the sequence Arg-Gly-Asp (RGD). This should be expected as a fundamental feature of both these immune responses is a change in the adhesive properties of the haemocytes. Sepharose beads coated with RGD peptides were more rapidly encapsulated by PL than beads coated with control peptides (Pech and Strand, 1995). A haemocyte aggregation inhibitor protein (HAIP), which prevents haemocyte aggregation in *M. sexta* has been isolated by Kanost and co-workers (Kanost *et al.*, 1994). This protein prevents haemocyte aggregation and may act as a negative regulator of inter-cell clumping *in vivo*. The biogenic amines and the hormone octopamine (Baines *et al.*, 1992; Dunphy and Downer, 1994) have also been reported to enhance nodule formation in insects. Thus the regulation of nodule formation and encapsulation appears to involve many endogenous factors. Given that these immune

responses comprise many steps, involving cell communication and changes in cell activity, it should be expected that many more factors will be identified that are involved in modulating these responses.

1.4 Mechanisms of immunosuppression by microbial pathogens

Although many successful insect pathogens and parasites avoid the immune responses due to non-recognition, many also disrupt the cellular immune responses. One of the fundamental properties of insect blood cells is the ability to attach and spread on a foreign surface. Many entomopathogens and parasites produce factors that inhibit haemocyte spreading, exemplifying its importance in defence reactions. Infection with *M. ansiopliae* or *B. bassiana* is accompanied by suppression of haemocyte spreading and pseudopodia formation and the subsequent inhibition of phagocytic activity (Hung *et al.*, 1993; Vilcinskas *et al.*, 1997b). Low molecular weight peptide toxins such as destruxins (Huxham *et al.*, 1989) and high molecular weight proteins such as proteases have been shown to have similar effects of the haemocytes (Griesh and Vilcinskas, 1998; Vilcinskas *et al.*, 1997b). Endoparasitic wasps, which inject their eggs into insect hosts, also inject calyx fluid that usually contains polydnaviruses and immunosuppressive factors (reviewed by Vinson, 1990 and Strand and Pech, 1995). Many of the studied parasitoid wasps suppress encapsulation by destruction of haemocytes (Rizki and Rizki, 1990) and inhibition of haemocyte spreading (Richards and Edwards, 2002; Stettler *et al.*, 1998).

Finally, some fungal pathogens have been found to use strategies such as molecular mimicry that allow them to avoid recognition within the host. *B. bassiana* blastospores lack a well-defined cell wall *in vivo* (unlike blastospores produced in culture) and have been shown to possess a surface coat that either mimics host components or is comprised of adsorbed hemolymph proteins (Pendland *et al.*, 1993). Unlike *in vitro* produced blastospores, the blastospores found *in vivo* cross react with antibodies against haemocyte lysate and they do not bind to a purified insect (*S. exigua*) haemolymph galactose-specific lectin. These changes in the fungal cell surface may explain why *in vivo* blastospores of *B. bassiana* are not readily recognised by host haemocytes, unlike blastospores produced in culture. Disguising strategies such as these are likely to be an important component in the pathogenesis of many entomopathogens.

1.5 Aims of the project

The insect cellular immune response has been studied in detail in many model insects and much has been written on the subject. The present work aimed to characterise further the cellular immune responses in the tobacco hornworm, *M. sexta* to bacterial and fungal infections. This insect provides large amounts of haemolymph for immunological assays and is therefore a useful model insect in the study of cellular immunity.

Both nodule formation and phagocytosis were assessed. Although previous studies have investigated the cellular immune responses of this insect (Horohov and Dunn, 1983), the advances in methodology and technology have enabled insect haemocyte morphology and function to be studied with increased detailed compared with earlier studies. Cytochemical analysis, improvements in microscopy, new *in vitro* protocols for haemocyte monolayers, the use of fluorescently labelled microorganisms and foreign particles are some of the recent advances that facilitate a better assessment of insect cellular immunity.

In the present study, I assessed the importance of phagocytosis and nodule formation during infection of *M. sexta* and investigated the role of haemocyte subpopulations in these responses. The effect of eicosanoids on the nodule response toward fungi was also investigated. An important property of the immune system is to react quickly to the presence of nonself. It is clear from previous studies that the insect immune system responds to nonself by changes in haemocyte populations and haemocyte behaviour. This hypothesis was tested during fungal and bacterial infection in *M. sexta* and following the injection of microbial products. Also investigated were the pathological effects of the fungal pathogen *B. bassiana* on the insect haemocyte morphology.

The pathogen side of the interaction with the cellular immune system was studied using the entomopathogenic bacterium *P. luminescens*. This highly virulent bacterium multiplies in the haemolymph of *M. sexta* with apparent disregard for the immune responses. It is not known how this pathogen overcomes phagocytosis and nodule formation and this was the subject of the present study.

Chapter 2

Materials and Methods

2.1 Labware and reagents

All reagents and labware were obtained from Sigma (Poole, Dorset) unless otherwise stated. Solutions used in all immunological assays were prepared with sterile Milli-Q water and their pH and osmolality adjusted to 6.5 and 310 mosmol/Kg respectively. Grace's insect medium was obtained from Sigma (G8142) and contained $NaHCO_3$ and L-glutamine.

Glassware in contact with insect haemocytes was previously soaked overnight in 1% (w/v) E-Toxa clean and then treated in accordance with Sigma Technical Bulletin 210 to deactivate and remove potential pyrogen contamination.

2.2 Maintenance and culture of experimental organisms

2.2.1 The tobacco hornworm *Manduca sexta*

Manduca sexta (Lepidoptera: Sphingidae) was reared in an insectary as described by Reynolds *et al.* (1985). Briefly, larvae were maintained individually at 25°C under a photoperiod of 17 hours light: 7 hours dark and fed on an artificial diet based on wheat germ. First instar larvae were placed on diet and incubated under the above conditions until the "wandering" stage. Larvae were then placed in drilled out wooden blocks for 10 days until pupation. Newly emerged adults were housed in a separate moth cage and given water and 10% glucose solution daily. Eggs were laid onto nappy liners impregnated with macerated tobacco leaves and were harvested daily. They were kept in individual pots until eclosion, and then placed on artificial diet to continue the life cycle. Day 0 (newly moulted) 5^{th} instar larvae were utilised for all experiments in this study (unless otherwise stated). The insects were kept under the above conditions in all cases.

Instars were identified by distinct morphological features such as spiracle size, coloration and head capsule slippage.

2.2.2 Entomopathogenic fungi

Metarhizium anisopliae isolate ME1 (ARSEF 2735) and *Beauveria bassiana* (ARSEF 304 and 2253) were obtained from the USDA-ARSEF collection held at the Plant Protection Research Unit, NY, USA. All isolates were grown on ¼-strength Sabouraud's dextrose agar supplemented with yeast extract (¼ SDAY; 1% dextrose, 0.25% mycological peptone, 1.5% agar, 0.5% yeast extract, pH 6.5) at 27°C for 10-14 days until conidiation. Conidia were harvested from these plates using a sterile loop and suspended in 0.05% (v/v) Tween 80. Conidial clumps and mycelial fragments were removed by passing the suspension through a 30-gauge syringe needle and then sonicating in an ultrasonic water bath for 30 sec. The conidial concentrations were determined using an improved Neubauer haemocytometer.

Master cultures of conidia were harvested (as above) from original plates and resuspended in 15% (v/v) glycerol. These suspensions were divided into 0.5 ml aliquots, flash frozen in liquid nitrogen and stored at - 20°C. Routinely, surface cultures were prepared from these master stocks by streaking onto ¼ SDAY plates and incubating under the conditions described above. All subcultures of fungi were made from these master plates (plates could be stored up to 2-3 months at 4 °C without a significant loss in conidia viability). The viability of the conidia was routinely assessed by overnight germination on ¼ SDAY plates.

2.2.3 Experimental Bacteria

Overnight (or longer) cultures of bacteria were frequently prepared throughout this study. *Escherichia coli* strain DH5α or SOLR (Stratagene) and *Staphylococcus aureus* strain H462 (obtained from Dr. E. Feil, University of Bath) were grown at 37°C in Luria-Bertani liquid broth (LB; 1% tryptone, 0.5% yeast extract; 1% sodium chloride, pH 7.0) with constant shaking. *Photorhabdus luminescens* strains TT01, K122 and W14 were obtained from Prof. R. ffrench-Constant (University of Bath) and grown at 27°C in 2 %

(w/v) proteose peptone number 3 broth (PP3, Becton Dickenson) with constant shaking. Long-term stocks of bacteria were maintained at - 20°C in 0.5 ml aliquots of 15% glycerol.

2.2.4 Preparation of *P. luminescens* filtrate

PP3 broth (10 ml) was inoculated with a loop of *P. luminescens* strains K122 and W14 and incubated at 27°C with shaking until the desired culture age was reached. The bacterial suspension was then removed by centrifugation at 1200 g for 10 min and the supernatant was filtered sequentially through a 0.45 μm and then a 0.22 μm membrane filter (Millipore). Bacterial filtrates were routinely streaked onto PP3 agar plates and incubated at 27 °C to check for contamination. The filtrates were stored for up to a week at 4 °C before use.

2.3 Microbial inoculation of *M. sexta*

2.3.1 Fungal inoculation by dipping

Larvae were immobilised on ice for 15-30 min prior to infection and then dipped into a conidial suspension (prepared as above to the desired concentration). Individual larvae were first cleaned to remove food and faecal matter. They were then held with forceps by their dorsal horn and completely submerged in the conidial suspension before being transferred to clean plastic containers. For the first 24 h post infection, larvae were maintained in individual pots at 25°C, 100% RH (relative humidity) in a sealed desiccator (to promote conidial germination) and without artificial diet. Removal from the diet over the first 24 h ensured the fungus could germinate without interference from antifungal agents in the diet. Subsequently, infected larvae were maintained under normal rearing conditions.

2.3.2 Inoculation by injection

Larvae were chilled on ice for 30 min and the cuticle surrounding the puncture site was disinfected with 70% ethanol. In all cases, larvae were injected with a conidial or

bacterial suspension between the 7th and 8th abdominal segment using a 30-gauge needle attached to a 25 µl Hamilton syringe. As with inoculation by dipping, larvae were maintained in individual pots under at 25°C and were not given food until 24 h post injection. Subsequently they were reared under normal culture conditions.

2.3.3 Plating out haemolymph from fungal-infected larvae

Haemolymph from infected larvae was assessed for the presence of the fungus at different times post infection. Larvae were infected with *B. bassiana* isolate 304 by dipping in a suspension of conidia ($2x10^7$/ml in Tween 80) as outlined in section 2.3.1. At successive days post-infection, larvae were chilled on ice for 30 min and surface-disinfected with 70% ethanol. The dorsal horn was cut and individual larvae were bled into sterile 1.5 ml polypropylene tubes under sterile conditions. The haemolymph was serially diluted (1:10, 1:100, 1:1000, 1:10000) in phosphate-buffered saline pH 6.5 (PBS, Sigma P4417) and 50 µl was spread onto ¼ SDAY plates (2 plates per larva). Plates were incubated at 27°C for up to 10 days and the resulting colony forming units were counted for each larva. Eight infected larvae were bled on each of 6 successive days post-infection.

2.4 Injection of *M. anisopliae* ME1-infected larvae with pharmacological agents

Arachidonic acid (AA) and the eicosanoid biosynthesis inhibitors dexamethasone (9α-fluoro-16α-methylprednisolone), esculetin (6,7-dihydroxycoumarin), ibuprofen (α-methyl-4-[isobutyl]-phenylacetic acid), indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), and phenidone (1-phenyl-3-pyrazolidone) were all obtained from Sigma.

2.4.1 Weight gain experiment

Larvae were initially injected with a range of doses of *M. anisopliae* ME1 conidia (3×10^6, 1×10^6, 3×10^5, 1×10^5, 3×10^4 or 1×10^4 in 15 µl Tween 80) and their weight gain was recorded daily. It was found that 3×10^5 conidia was a suitable dose for assessing the weight gain of infected larvae injected with various pharmacological agents.

Larvae were chilled on ice and injected with the pharmacological agents using the injection protocol described in section 2.3.2. All pharmacological agents were dissolved in 95% ethanol and injected into test larvae in 5 µl aliquots. Each larva received 10 µg of eicosanoid biosynthesis inhibitor. AA was injected at a dose of 50 µg per larva together with dexamethasone. The larvae were left for 15 min to allow the drugs to circulate within the haemocoel before conidia were injected into the opposite side of the body. Larvae were injected with 3×10^5 ME1 conidia suspended in 15 µl of 0.05% Tween 80. Control larvae were injected with 0.05% Tween 80 alone.

Other control groups of larvae were set up in which the inhibitors were injected without the fungus to determine whether the drug had any visible detrimental effects alone. A further control group received conidia and solvent (95% ethanol) without the inhibitor. After injection, larvae were held without food for 24 h and then replaced on artificial diet and maintained individually under normal rearing conditions. The insects were assessed daily for weight gain and symptoms of infection and death.

2.4.2 Mortality experiment

The effect of dexamethasone on larval mortality caused by mycosis was assessed. As with the weight gain experiment, larvae were initially injected with a range of conidial doses (3×10^4, 1.5×10^4, 7.5×10^3, 4.5×10^3, 1.5×10^3 in 15 µl 0.05% Tween 80) to find a suitable dose for this assay. Control larvae were injected with 0.05% Tween 80 alone. Mortality was recorded when the larva was unable to right itself when placed on its dorsal side and was unable to respond to prodding. A dose of 4.5×10^3 conidia was determined to be suitable.

Larvae were injected with 10 µg dexamethasone, 10 µg dexamethasone plus 50 µg AA or 95% ethanol alone (in 5 µl volume) as outlined in the weight gain experiment. They were left for 15 min to allow the drugs to circulate within the hemocoel before injection with 4.5×10^3 ME1 conidia. Individuals, incubated as above were then checked daily for mortality using the above criteria.

2.5 Fungal growth and germination in the presence of dexamethasone

Conidial germination and hyphal growth of *M. anisopliae* ME1 was assessed in the presence of dexamethasone *in vitro*. ¼ SDAY was sterilised by autoclaving and allowed to cool. Just before the agar had set, dexamethasone (dissolved in 95% ethanol) or 95% ethanol alone was added to the medium. The final concentration of dexamethasone in the agar medium was 85 μM, the same as that used in the *in vitro* microaggregation assay. A conidial suspension was prepared (as above) to a concentration of 7×10^5 / ml and this was spread onto the surface of the medium containing the dexamethasone. After 12 h at 27°C, germination and germ tube elongation of the fungus were assessed at x 200 magnification using an Olympus BH-2 microscope with phase contrast optics. Fields of view were captured using a Nikon Coolpix 950 digital camera and the images were assessed on a PC using Adobe Photoshop graphics software.

2.6 *In vivo* nodulation assay

Individuals injected with various microbial suspensions (see below) were immobilised on ice for 30 min before dissection under 1% (w/v) NaCl solution saturated with phenylthiourea (which prevented general post-dissection melanization). Larvae were dissected by removing the head and cutting along one side of the body to reveal the haemocoel. They were then pinned out on a wax block and the melanised nodules within the haemocoel were counted using a stereomicroscope. After initial counting, the digestive tract was excised allowing previously unexposed nodules to be counted (as described by Miller *et al.*, 1994).

2.6.1 Nodulation of *M. anisopliae* ME1 conidia in larvae injected with pharmacological agents

Pharmacological agents were injected into larvae using the same protocol outlined in the mortality assay (section 2.4.2). Dexamethasone (10 μg), dexamethasone (10 μg) + AA (50 μg) or 95% ethanol alone were all injected in a 5 μl volume. Injected larvae were held for 15 min at room temperature to allow the drugs to circulate within the haemocoel. They were then injected with 30,000 *M. anisopliae* ME1 conidia suspended in 15 μl

0.05% Tween 80. Individuals were left under normal rearing conditions for 24 h and nodule formation was then assessed as outlined above.

2.6.2 Influence of bacterial species on nodule formation *in vivo*

Larvae were injected with different doses of *E. coli* (SOLR) to find a suitable dose for the *in vivo* nodulation assay. A dose of 1 x 10^6 bacterial cells was adequate to induce good levels of nodulation. However, the volume in which the bacteria were suspended was important when it came to counting the nodules. In a 10 µl volume, most of the nodules were located at or near the injection site and were difficult to count. Therefore, 1 x 10^6 bacteria were injected in 100 µl resulting in a wider spread of the nodules within the haemocoel.

Individuals were injected with 1 x 10^6 *E. coli* (SOLR) or *P. luminescens* (W14 or K122) in 100 µl Grace's insect medium (GIM) and incubated for 1, 4 and 24 h. At these times, insects were dissected and the numbers of nodules counted as described above. Comparisons were made between live and heat-killed bacteria (killed by autoclaving at 121°C for 15 min) and the different bacterial types. Two groups of control larvae were set up consisting of untreated and GIM-injected individuals.

2.6.3 Nodulation of *E. coli* following injection of *P. luminescens* filtrate

A 48 h culture filtrate was produced for *P. luminescens* W14 and K122 as outlined in section 2.2.4. Test larvae were injected with 10 µl of filtrate (or GIM for control larvae) and incubated at room temperature for 15 min. Larvae were then injected into the opposite side of the body with 100 µl of 1 x 10^6 heat-killed *E. coli* and nodule formation was assessed at 1, 4 and 24 h post-injection as previously described. The effect of heat on W14 filtrate was also assessed by boiling 1 ml aliquots of filtrate in a water bath for 3 min. Boiled filtrate was subsequently injecting into test larvae with the bacteria.

2.7 Haemocyte monolayers of *M. sexta*

2.7.1 Optimisation of monolayer formation

Many insect immunological assays involve the preparation of haemocyte monolayers in which haemocytes are encouraged to adhere to a glass surface. Many methods are available but each is often specific to the insect being used and the particular assay being carried out.

For this reason monolayer formation was optimised for the haemocytes of *M. sexta*. Many different parameters were evaluated and changed according to the quality of the monolayer produced. The finalised method is given here.

Newly moulted 5th instar larvae were chilled on ice for 30 min. This step anaesthetised the larvae and minimised subsequent coagulation and melanisation of the haemolymph. Individuals held at 4°C were surface disinfected with 70% ethanol and their dorsal horn was cut at its midpoint using clean dissection scissors. Two different methods of monolayer formation were employed depending on the desired monolayer type: haemocytes were either washed or unwashed before monolayer formation.

2.7.2 Washing method of monolayer formation

Insects were bled as described above and 100 µl haemolymph was dripped directly into sterile 1.5 ml polypropylene tubes containing 900 µl of ice-cold saline solution with the composition 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2.6H_2O$, 1.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 192.8 mM sucrose, pH 4.5. This is a modification of *Manduca* physiological saline solution (i.e. Calcium free and low pH to minimise coagulation). The tubes were quickly inverted, mixing the haemolymph with the buffer, and the haemocytes were sedimented by centrifugation at 170 g for 8 min at 4°C. The cell pellet was gently resuspended in 200 µl ice-cold GIM using a Gilson pipette with a wide bore pipette tip. At this point suspensions were usually pooled. An improved Neubauer haemocytometer was used to adjust the cell density of the suspension to 6 x 10^5 cells/ml and this was kept in a chilled polystyrene bijou tube until required.

Monolayers were made by pipetting 100 µl of the haemocyte suspension onto 10 mm diameter round coverslips (Scientific Lab Supplies Ltd, MIC3330) at the bottom of a well in a 24-well plate (Fahrenheit, Milton Keynes, UK, 3226). The haemocytes were incubated at room temperature (22-25°C) for 30 min, allowing them to settle and attach to the coverslip. After this, 500 µl GIM (at room temperature) was added to the well and the cells were incubated for a further 20 min. Finally, the monolayer was gently rinsed twice in 500µl GIM to remove any unattached cells and placed in a new well with fresh GIM until required.

2.7.3 Unwashed method of monolayer formation

Monolayer formation using washed haemocytes ensured reduced interference from components of the plasma. A quicker method of monolayer formation, that required no centrifugation step, was employed in some of the assays described in this study. Approximately 50 µl of haemolymph was dripped directly into 1 ml of ice-cold GIM in a sterile 1.5 ml polypropylene tube. This was inverted and the haemocyte concentration was adjusted to 6 x 10^5/ml as above. One hundred µl of this suspension was pipetted onto a 10 mm round coverslip onto which the haemocytes settled and adhered. Cells were incubated at room temperature for 20 min after which the medium was replaced with fresh GIM and incubated for a further 30 min. The monolayers were rinsed in GIM to remove unattached cells and transferred to a new well containing fresh GIM.

2.7.4 Haemocyte viability

Haemocyte viability was regularly assessed by trypan blue exclusion. Monolayers were incubated in 0.2% (w/v) trypan blue in PBS for 10 min at room temperature. The trypan blue was removed by rinsing the monolayers in fresh PBS and the cells were visualised by phase contrast microscopy; live cells excluded the dye while dead cells stained blue.

2.8 Staining haemocyte monolayers

2.8.1 Labelling the haemocyte cytoskeleton with phalloidin

Haemocyte monolayers were prepared on 10 mm round glass coverslips and kept in the wells of a 24-well plate as described above. Monolayers were washed once in PBS and then fixed in 4 % (w/v) formaldehyde (made fresh by dissolving paraformaldehyde in PBS) for 15 min at room temperature. After a further 3 washes, the monolayers were incubated for 10 min in 0.285 M NH_4Cl. This step acted to quench the autofluorescence of the formaldehyde for subsequent fluorescent microscopy. Cells were permeabilised in 0.2% (v/v) Triton X-100 for 5 min, washed and then stained with fluorochrome-conjugated phalloidin (fluorescein isothiocyanate [FITC] or tetramethylrhodamine isothiocyanate [TRITC]). For each coverslip, a 60 µl drop of phalloidin solution (0.1 µg/ml, final concentration in PBS) was applied to a strip of parafilm and the coverslip was inverted onto the drop. Cells were stained at room temperature for 40 min in the dark and then washed 4 times in PBS and twice in dH_20. Monolayers were mounted overnight at room temperature in 5 µl Mowiol-4-88 solution (Calbiochem) and stored at 4°C.

2.8.2 Staining haemocyte nuclei with propidium iodide

Monolayers were often dual stained with phalloidin and the nucleic acid stain propidium iodide. After labelling with the phalloidin as described, the cells were washed twice in PBS and then treated with ribonuclease A (RNase). This step was essential as haemocyte RNA labelled strongly with the propidium iodide resulting in diffuse staining of the cell. Prior to use, the RNase solution (0.3 mg/ml, final concentration in dH_20) was boiled for 10 min to remove any residual DNase and incubated with the monolayer for 30 min. Monolayers were washed twice in PBS and stained with propidium iodide (1 µM, final concentration in PBS) for 10 min at room temperature. Monolayers were washed and mounted as described for phalloidin staining.

2.8.3 Antibody staining of plasmatocytes

Monoclonal antibody mAb MS13 (class IgG2b) was obtained as a gift from Drs D. Levin and M. Kanost (Kansas State University, USA). This monoclonal was generated in mouse as described by Willot *et al.* (1994) and served as a marker for the plasmatocyte class of haemocyte in *M. sexta*. Briefly, BALB/c mice were injected with a suspension of *M. sexta* haemocytes and approximately 7 weeks later, spleen cells were harvested and fused with X63Ag8.653 myeloma cells. Fused cells were cultured *in vitro* and their supernatants were tested by ELISA and immunofluorescence microscopy of fixed haemocytes. Monoclonal antibody MS13 was found to specifically label plasmatocytes.
In the present study, the protocol for indirect immunofluorescence was modified from that given by Wiegand *et al.* (2000). Monolayers were fixed in 2 % (w/v) formaldehyde in PBS (made fresh from paraformaldehyde) for 15 min. Cells were washed twice in PBS and then blocked in 3 % (w/v) bovine serum albumin (BSA) for 1.5 h at room temperature. The blocking solution was washed off and the monolayers were incubated overnight at 4 °C in hybridoma supernatant containing mAb MS13, without dilution. Cells were washed twice in PBS and then incubated for 20 min in the dark with secondary antibody solution (TRITC-conjugated goat anti-mouse IgG, Sigma T5393) at 1:100 (v/v) dilution in 0.3% BSA/PBS. After 4 further washes in PBS and once with dH_20, coverslips were mounted overnight in Mowiol as outlined previously. Control slides were incubated with the secondary antibody without primary labelling with mAb MS13.

A problem was encountered with the secondary antibody solution, which was found to give non-specific staining of *M. sexta* haemocytes even in the absence of the primary mAb MS13. Therefore, affinity purification of the secondary antibody was necessary. This was achieved by prior incubation of the secondary antibody solution with a strip of nitrocellulose coated with haemocyte proteins. The method employed is essentially that described by Wiegand *et al.* (2000). In the present study, a 1 ml haemocyte suspension (1 x 10^7/ml) prepared in ice-cold anticoagulant buffer (0.098 M NaOH, 0.146 M NaCl, 0.017 M EDTA, 0.041 M citric acid, pH 4.5, 450 mosmol/Kg) was ultrasonicated for 30 sec to lyse the cells. The lysate (1 ml) was vortexed and incubated with a 0.5 x 3 cm strip of nitrocellulose (Amersham) in a 1.5 ml polypropylene tube on a shaker for 30 min. The

nitrocellulose was rinsed with PBS and then blocked with 5% (w/v) milk powder (Marvel) in PBS for 30 min on a shaker. The nitrocellulose was rinsed again in PBS and incubated with the secondary antibody solution for 30 min with shaking. Following this, the strip of nitrocellulose was removed and the remaining secondary antibody solution was used for immunofluorescence.

2.8.4 Anti-α-tubulin immunofluorescence

Fixed and blocked monolayers were prepared as above (2% formaldehyde for 15 min; 3 % BSA for 1.5 h) and permeabilised in a PBS solution containing 0.285 M NH_4Cl and 0.2% Triton X-100 for 10 min. After 2 washes in PBS, the monolayers were labelled with a mouse anti-α-tubulin monoclonal antibody (1:2000 dilution, Sigma T5168) for 2 h at room temperature. Secondary antibody staining was then carried out as in section 2.8.3.

2.9 Fluorescence and confocal microscopy

Stained or phagocytosing haemocytes were examined using a Zeiss LSM-510 laser scanning confocal microscope or an Olympus BH-2 microscope fitted with epifluorescence optics. The Olympus microscope was frequently used when high resolution of the cell staining was not necessary as with cell counts or assessing levels of phagocytosis. The optical specifications for this microscope depended on the fluorochrome being viewed (FITC: 530 nm barrier filter, blue dichroic mirror, blue excitation filter; TRITC: 610 nm barrier filter, green dichroic mirror and green excitation filter).

The confocal microscope was interfaced with a PC running Zeiss LSM software that allowed control over all optical parameters. This included the type and intensity of the lasers and the type of objective lens being used. FITC was excited with an argon-ion laser (488 nm emission wavelength) and was detected using Zeiss LSM-510 filter set 09. TRITC and propidium iodide was excited using a Helium-Neon laser (543 nm emission wavelength) This microscope gave high resolution of stained haemocytes and enabled accurate quantification of ingested fluorochrome-labelled particles.

2.10 Scanning electron microscopy

Monolayers were prepared for scanning electron microscopy by fixing in 2% glutaraldehyde for 30 min at room temperature. They were washed twice in PBS and then incubated in 1% (w/v) osmium tetroxide in PBS for 30 min. After a double wash in dH_20 the cells were sequentially dehydrated in increasing concentrations of acetone (50% through to 100% in dH_20) and then critical point dried for 15 min to remove the acetone. Coverslips, mounted on SEM holders were sputter-coated with gold and viewed using a JEOL JSM-6310 electron microscope.

2.11 Monolayers from challenged larvae

The haemocyte profile of larvae challenged with various microorganisms or elicitors was assessed by monolayer formation at different times post-treatment. All monolayers were labelled with FITC-phalloidin and viewed by confocal microscopy.

2.11.1 Fungal infection

M. sexta larvae (day 0, 5^{th} instar) were infected with *B. bassiana* isolate 304 by dipping in a conidial suspension (2 x 10^7/ml in Tween 80) as outlined in section 2.3.1. Control larvae were dipped in Tween 80 alone. Individuals were bled on successive days post-infection and monolayers were made using the washing method (section 2.7.2). Monolayers were stained with FITC-labelled phalloidin and viewed by confocal microscopy. Similar experiments were performed with larvae infected with the fungi *B. bassiana* 2253 and *M. anisopliae* ME1 (using the same concentration of conidia).

2.11.2 Injection of larvae with other elicitors

Monolayers were also prepared from larvae challenged with other elicitors. Laminarin was injected in a 25 µl volume at 0.5% (w/v) in GIM. *E. coli* DH5α was injected in a 50 µl volume of GIM containing 5 x 10^7 bacterial cells. *P. luminescens* W14 was injected in a 10 µl volume of GIM containing approximately 70 bacterial cells. Control larvae were injected with GIM alone. All larvae were injected using the injection protocol outlined in

section 2.3.2. Monolayers were prepared at various time points post-injection and labelled with phalloidin.

The effect of wounding on the haemocyte profile was assessed in a separate experiment. Larvae were surface sterilised with 70% ethanol and pricked by proleg puncture with a sterile 30-gauge needle. They were then maintained in individual sterile pots and monolayers were made from separate larvae on successive days post treatment.

2.11.3 Monolayers from *M. sexta* larvae parasitized with *Cotesia congregata*

Pupae of the braconid parasitoid wasp, *C. congregata*, were obtained as a gift from Prof. N. Beckage (University of California, CA, USA). After adult wasps had emerged, they were incubated with *M. sexta* larvae (2^{nd} and 3^{rd} instar) in enclosed containers for 7 days and fed on a diluted honey solution. Wasps and larvae were then anaesthetised with CO_2 and the larvae were transferred to separate plastic pots containing artificial diet. Monolayers were made from late-stage parasitized larvae (when the final instar parasitoid larvae had started to emerge through the host cuticle).

2.12 Spreading of haemocytes on glass coverslips

Haemocyte suspensions were prepared as outlined in section 2.7.2 to a concentration of 6 x 10^5/ml in GIM and 100 µl of this suspension was pipetted onto clean glass coverslips. Haemocyte spreading on the glass surface was halted (after 2, 6, 10, 60, 120 min) by the addition of 4% (w/v) formaldehyde to the well. Haemocytes were subsequently fixed for 15 min at room temperature and then stained with FITC-phalloidin. The diameters of the haemocytes were measured on the confocal microscope using Zeiss LSM-510 software.

2.13 Incubation of monolayers with fungal elicitors

It was discovered that monolayers from *B. bassiana* 304-infected insects exhibited a new haemocyte profile. Attempts were made to induce this profile *in vitro* by incubation of healthy monolayers with fungal elicitors. The haemocyte profile of all the treated monolayers described in this section was assessed by confocal microscopy.

2.13.1 Conidia

Forty µl of washed *B. bassiana* 304 conidia in GIM (prepared as in section 2.2.2) at a concentration of 10^7/ml were added to washed monolayers in the wells of a 24-well plate. Monolayers were incubated for different lengths of time with the conidia and then fixed and stained with phalloidin.

2.13.2 *B. bassiana* 304 filtrate

A conical flask containing 100 ml ¼ SDAY broth was inoculated with 1 ml of 0.05% Tween 80 containing 10^6 conidia. This was incubated for 12 days at 27°C on a rotary shaker. The fungal suspension was centrifuged (1200 g for 15 min) and the supernatant passed through a double layer of filter paper (Whatmann, No. 1). Any remaining fungal fragments and conidia were removed by sequential filtration under vacuum (through a 1.0 µm then a 0.22 µm membrane filter). The resulting filtrate was stored for up to 1 week at 4°C. Monolayers were exposed to 10, 20 and 50% filtrate in GIM for 2 h at room temperature and were then fixed and phalloidin stained.

2.13.3 Infected plasma

Plasma was obtained from *B. bassiana* 304-infected larvae at different time points post-infection. Infected larvae (infected by dipping) were chilled and bled into an ice-cold polypropylene tube containing a few crystals of phenylthiourea. The cells were sedimented at 1200 g for 10 min and the supernatant was filtered through a 0.22 µm membrane filter. Washed haemocyte monolayers (prepared as above) were exposed to the infected plasma at 10% and 20% concentrations in GIM for 2 h at room temperature. Control monolayers were exposed to non-infected plasma. The monolayers were then fixed and phalloidin stained.

2.13.4 Laminarin

Monolayers were exposed to a range of concentrations of laminarin. A solution of laminarin, 0.5% (w/v) in GIM, was prepared and different volumes of this solution (15,

25, 50, 100 µl) were added to GIM to a final volume of 1 ml. The monolayers were exposed to the laminarin solutions for 2 h and 24 h. They were then labelled with FITC-phalloidin and viewed with phase contrast and confocal microscopy.

2.13.5 Coating coverslips in *B. bassiana* 304-infected plasma

Healthy and *B. bassiana* 304-infected larvae, at different stages post-infection, were bled and plasma obtained as described above. The plasma was chilled and 100 µl of neat and 50% diluted plasma in GIM was pipetted onto 10 mm round coverslips. These were left for 1 h at 4 °C then the plasma was removed by pipetting. Monolayers from healthy larvae were prepared on these coated coverslips using the protocol given in section 2.7.2.

2.14 Microaggregation of haemocytes *in vitro*

Microaggregation of haemocytes *in vitro* was used as an index of nodule formation. Many protocols were tested and the final protocol is given here in some detail. The method is similar, but not identical to that independently developed by Miller and Stanley (2001). Because haemocyte aggregation *in vitro* is sensitive to the presence of foreign particulates, all manipulations were carried out in a laminar flow workstation. In addition, all equipment, solutions and test larvae were kept ice-cold prior to use, and all haemocyte suspensions were handled with care to minimise cell lysis that could initiate coagulation. Larvae were chilled on ice for 45 min and the area around the dorsal horn was disinfected with 70% ethanol. This step also cleaned the cuticle of any particulate matter, which could contaminate the haemolymph. The dorsal horn was cut at its midpoint using clean dissection scissors and approximately 100 µl of haemolymph was dripped into 1.5 ml polypropylene tubes containing 1 ml of ice-cold solution with the composition 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2.6H_2O$, 1.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 192.8 mM Sucrose, pH 4.5. This is a modification of *Manduca* physiological saline solution (i.e. Calcium free, low pH). The tubes were quickly inverted, mixing the haemolymph with the buffer, and the haemocytes were sedimented by centrifugation at 400 g for 8 min at 4°C. The cell pellet was gently resuspended in 200 µl cold GIM and suspensions were pooled. An improved Neubauer haemocytometer was used to adjust the cell density of the

suspension to 1 x 10^6 cells/ml and this was kept in a chilled polystyrene bijou tube until required.

A suspension of *M. anisopliae* conidia in GIM was prepared containing 5 x 10^7 conidia/ml. 100 µl of conidial suspension was mixed with 200 µl of haemocyte suspension in a 0.5 ml polypropylene tube that had previously been siliconised overnight using Sigmacote (Sigma, Sl-2). This tube was incubated for 15 min on a tube rotator at slow speed (1 rpm) and at room temperature. After incubation, the cell suspension in each tube was transferred to one well of a 96-well microtitre plate and the microaggregates were either counted immediately using a Nikon TMS-F inverted microscope or fixed with 4% (w/v) formaldehyde and viewed later. Microaggregates of 5 or more cells were counted as incipient nodules.

2.14.1 Microaggregation in the presence of eicosanoid biosynthetic inhibitors

Five µl of test solution (in 95% ethanol) was added to the *Metarhizium anisopliae* ME1 conidial suspension, vortexed and then mixed with the haemocyte suspension (outlined above). Haemocytes were exposed to 85 µM dexamethasone alone, 2.7 µM AA alone, or to both agents together (i.e. final concentration of 85 µM dexamethasone plus 2.7 µM AA). As a control for the solvent, 5 µl 95% ethanol was also tested. The haemocyte suspension was incubated under the above conditions and the resulting microaggregates were counted for each treatment.

2.15 Toxicity of dexamethasone and AA *in vitro*

To investigate the effects of dexamethasone and AA in the *in vitro* haemocyte microaggregation assay, it was necessary to test the toxicity of these compounds to haemocytes *in vitro*. Haemocyte monolayers were prepared by pipetting 100 µl haemocyte suspension (prepared using the same procedure as that in the *in vitro* microaggregation assay) onto 10 mm round glass coverslips. These were kept in the wells of a 24-well plate (Falcon). The monolayers were exposed to a range of AA and dexamethasone concentrations for 15 min at room temperature. Cells were then washed and stained with 0.2% (w/v) trypan Blue (in PBS) for 15 min to assess viability. They

were then washed twice in GIM and viewed on an Olympus BH-2 microscope. The proportion of dead cells was recorded over 10 fields of view.

2.16 Phagocytosis assay of *M. sexta* haemocytes *in vitro*

An *in vitro* phagocytosis assay using monolayers (prepared as above) was developed for *M. sexta* haemocytes. The method was modified from that given by Rohloff *et al.* (1994) and Richards and Edwards (2000).

2.16.1 Preparation of FITC-labelled yeast and bacteria

E. coli (DH5α or SOLR), *S. aureus* (H462) and *P. luminescens* (various strains) were grown overnight in liquid media as described in section 2.2.3. The bacterial suspensions were centrifuged at 2000 g for 15 min and the cell pellet was resuspended in sterile PBS. The bacteria were washed a further 2 times before being killed by heating for 20 min at 95°C. After an additional wash in PBS, the cell pellet was resuspended in 10 ml 0.2 M carbonate-bicarbonate buffer pH 9.4 to a final concentration of about 1 x 10^9/ml.

One mg of FITC (Isomer I) was added to the bacterial suspension (0.1 mg/ml final concentration) and the bacteria were subsequently stained in the dark for 1 h with shaking at 27°C. They were then washed a minimum of 6 times in PBS as before, or until no FITC remained in the supernatant. The cell pellet was finally resuspended in 10 ml GIM and this was separated into 0.5 ml aliquots and stored in the dark at -20°C.

Saccharomyces cerevisiae was grown overnight in 20 ml YPD medium (1% yeast extract, 1% bacteriological peptone, 2% dextrose, pH 7.0) at 26°C with constant shaking. The staining procedure was similar to that of the bacteria except that the yeast cells were centrifuged at 700 g for 15 min and prepared to a final concentration of 1 x 10^7/ml.

2.16.2 *In vitro* phagocytosis assay

Many *in vitro* phagocytosis assays with insect haemocytes utilise FITC-labelled yeast cells (*e.g.* Rohloff *et al.*, 1994) as the phagocytic particles. This was attempted with *M.*

sexta haemocytes in the present study but the level of phagocytosis of the yeast was very low compared with that seen with bacteria. Therefore, FITC-labelled *E. coli* was used for all standard phagocytosis protocols described in this report.

Monolayers, prepared from a single haemocyte suspension (prepared as in section 2.7) were covered with 500 µl GIM in the wells of a 24-well plate. Forty µl of GIM, containing 4 x 10^6 FITC-labelled bacteria, was added to each monolayer and incubated in the dark for 2 h at 27°C. After the incubation, monolayers were gently rinsed with GIM to remove any un-attached bacteria. They were then transferred to a well containing 0.2% (w/v) trypan blue in PBS. Trypan blue quenches the fluorescence from un-phagocytosed (extracellular) bacteria while the bacteria that have been phagocytosed remain fluorescent (Hed, 1986). After 20 min, the trypan blue was removed with 2 further washes in PBS and the monolayers were assessed by fluorescence microscopy as described in section 2.9. In most cases, phagocytosis was assessed by determining the percentage of phagocytic haemocytes within the monolayer i.e. those that had ingested 1 or more bacteria.

For all phagocytosis experiments outlined below, the viability of haemocytes was routinely assessed by trypan blue exclusion.

2.16.3 Assessing the effects of eicosanoid biosynthetic inhibitors on phagocytosis

Prior to the addition of FITC-labelled *E. coli*, haemocyte monolayers were exposed to the eicosanoid biosynthetic inhibitors dexamethasone and ibuprofen. The inhibitors were dissolved in 95% ethanol and 5 µl of this was added to 495 µl GIM to give final concentrations of 50 µM ibuprofen and 50 µM dexamethasone. Monolayers were incubated in the inhibitor solutions for 20 min before the addition of FITC-labelled *E. coli* as outlined above. Phagocytosis of the bacteria was assessed after 2 h by counting the percentage of phagocytic haemocytes on the monolayer.

2.16.4. Incubation of haemocytes with 48 h *P. luminescens* culture filtrate

P. luminescens strain W-14 was grown in liquid medium for 48 h and a filtrate produced as described in section 2.2.4. Filtrates were also made from *E. coli* (strain SOLR, Stratagene) in a similar fashion. Monolayers were incubated in the presence of the filtrates (diluted 1:10 in GIM) for 5 min and then a standard phagocytosis assay was performed by adding FITC-labelled *E. coli*. Control monolayers were exposed to PP3 broth alone (1:10 dilution in GIM) or remained untreated. The percentage of phagocytosing haemocytes was counted for each treatment over 10 fields of view.

One ml fractions of *P. luminescens* W-14 filtrate (48 h) were also boiled for 3 min and these was tested for effects on phagocytosis. Controls included non-heated 48 h filtrate and heated and non-heated PP3 broth (all were diluted 1:10 in GIM).

2.16.5 Dose response of W-14 filtrate on phagocytosis

Haemocytes were exposed to a range of concentrations (0-10% in GIM) of 48 h culture filtrate from *P. luminescens* W-14. The filtrates were made in the same manner as described in section 2.2.4. As above, monolayers were exposed to the filtrate solutions for 5 min before the addition of the FITC-*E. coli* and initiation of phagocytosis.

2.16.6 Age of W-14 and K-122 culture filtrate and effects on phagocytosis

Large scale shake cultures were set up for strains W-14 and K-122 by inoculation of 250 ml of 2% PP3 broth in 500 ml conical flasks. The cultures were incubated at 27°C with shaking and at selected time points (2, 4, 8, 12, 36, 48 h), 20 ml of the culture was removed under sterile conditions. The optical density (at 600 nm) of the bacterial culture at each time point was recorded and then filtrates were prepared from each suspension as described in section 2.2.4.

The filtrates were stored at 4 °C until required for the phagocytosis assay. All filtrates were diluted 1:10 in GIM and then exposed to monolayers for 5 min as above before the addition of the bacteria. Control monolayers were incubated with bacteria and GIM only.

2.16.7 Phagocytosis of *P. luminescens*

Haemocytes were assessed for their ability to phagocytose FITC-labelled *P. luminescens* cells. *P. luminescens* strains K-122 and W-14 were cultured for 48 h in 100 ml of PP3 broth at 27°C with shaking. The 2 cultures were divided in half and one was autoclaved. Both fractions (of live and dead cells) were stained with FITC as outlined previously. The same procedure (heat-killed *vs.* live) was also applied to *E. coli* strain SOLR. Autoclaved and non-autoclaved bacterial suspensions were streaked onto agar medium to check for viability.

All 6 FITC-labelled bacterial suspensions were adjusted to a cell concentration of 1 x 10^8/ml in GIM and 40 µl of this was applied to monolayers. A phagocytosis assay was performed using the standard protocol.

2.16.8 Phagocytosis in the presence *P. luminescens*-infected plasma

Newly moulted 5th instar larvae were infected by injection with a suspension of *P. luminescens* W-14 bacteria. The bacterial cells were obtained from a 24-hour culture and prepared to a final cell concentration 1 x 10^7/ml in GIM. Using the injection procedure described in 2.3.2, larvae were injected with 100 µl of this *P. luminescens* suspension and incubated with artificial diet under normal rearing conditions.

Infected insects (at late stages of infection) were chilled on ice for 30 min and bled into a chilled 1.5 ml polypropylene tube containing a few crystals of phenylthiourea. The haemolymph was then centrifuged at 1200 g for 10 min to sediment the haemocytes and bacteria and the plasma supernatant was removed. The plasma was filtered through a 0.22 µm membrane filter (Millipore) and stored on ice. Control plasma was obtained in a similar fashion from uninfected larvae of the same age.

Infected and control plasma (diluted 1:10 in GIM) was exposed to haemocyte monolayers for 5 min. Following this, 40 µl FITC-labelled *E. coli* was then added to the monolayers and a standard phagocytosis assay was performed. True control monolayers were exposed to GIM alone.

2.16.9 *In vitro* phagocytosis of different particles

Phagocytosis was assessed in vitro with different particles other than E. coli. Monolayers were incubated with (a) FITC-labelled *S. aureus* (b) Highly positively and negatively charged 1 μm fluorescent polystyrene microspheres (Molecular Probes, see section 2.17). The assay was performed as outlined above. Thus, monolayers on glass coverslips were exposed to 4 x 10^6 particles in a 40 μl volume of GIM. They were incubated with the particles for 2 h at 27°C and then the extracellular particles were quenched with trypan blue. Haemocytes were stained with TRITC-phalloidin and viewed by confocal microscopy.

It was found that the fluorescent microspheres were not quenched by the trypan blue. Thus, confocal microscopy alone was used to determine whether these particles had been ingested or not.

2.17 *In vivo* phagocytosis assay

Newly moulted 5^{th} instar larvae were chilled on ice for 30 min and then injected with a suspension of fluorescent particles (bacteria or beads). *E. coli* and *S. aureus* were labelled with FITC using the staining protocol outlined in section 2.16.1. Two types of fluorescent microsphere (1.0 μm diameter, Molecular Probes) were used with different surface properties (i) green, amine-modified (highly positively charged) and (ii) red, carboxylate-modified (highly negatively charged). Insects were injected with a suspension of 5 x 10^7 fluorescent particles (bacteria or beads) and incubated for 4 h in the dark. Injected insects were bled and monolayers prepared as described in section 2.7.3 Monolayers were fixed and labelled with TRITC-phalloidin as outlined above. The monolayers from insects injected with red fluorescent microspheres were labelled with FITC-phalloidin. All slides were kept in the dark at 4°C until required. Phagocytosis was assessed by confocal and fluorescent microscopy.

A time course of phagocytosis *in vivo* was completed using FITC-labelled *E. coli*. Insects were injected with the bacterial suspension as outlined above and incubated for 30 min, 2, 6 or 24 h. Monolayers were prepared from injected larvae at the different time points (4

larvae per time point; 2 monolayers per larva). The monolayers were labelled with phalloidin as above and assessed by fluorescent microscopy only.

2.17.1 *In vivo* phagocytosis in larvae of different ages

M. sexta was found to possess a class of haemocyte with high phagocytic ability. With the following exception, all experiments in characterising this haemocyte type were done with day 0 5th instar larvae. However, larvae of different developmental stages were used to find out whether this result was specific to newly moulted 5th instars. FITC-labelled *E. coli* was injected into larvae of 4 different stages (final-day 3rd instar, mid-4th instar, final-day 4th instar, wandering stage). The amount injected was dependent on the larval size (working with the ratio 5 x 10^7 bacteria per 1.2 g body weight). Larvae were then incubated as above for 4 h and monolayers were made and labelled with TRITC-phalloidin.

2.18 Phagocytosis *in vitro* and *in vivo* in 4 other Lepidoptera

M. sexta was not the only insect used in this study. *Spodoptera littoralis*, *Spodoptera exigua*, *Lacanobia oleracea* (all Noctuidae) and *Galleria mellonella* (Pyralidae) were all used for the assessment of phagocytosis by haemocytes *in vitro* and *in vivo*. *S. littoralis*, *S. exigua* and *L. oleracea* were obtained from the Central Science Laboratory, York. *Galleria mellonella* was obtained from Live Food UK, Somerset. Final-instar larvae were used for all experiments.

All insects were chilled on ice for approximately 20 min and were then injected with a suspension of FITC-labelled *E. coli* (1 x 10^9/ml). Insects were injected in a similar manner as the *M. sexta* larvae except the injected volume was different due to body size (*G. mellonella* 10 µl; *S. littoralis* and *L. oleracea* 20 µl; *S. exigua* 5 µl). Injected insects were incubated at 27 °C in the dark for 4 h after which time they were bled and monolayers made.

Insects were chilled on ice for 20 min and their cuticle was disinfected with 70% ethanol. They were bled by proleg puncture into 1.5 ml polypropylene tubes containing 1 ml of

ice-cold GIM, pH 6.5. The tube was inverted twice and the haemocyte concentration was adjusted to 5 x 10^5/ml. One hundred µl of this suspension was pipetted onto 10 mm diameter glass coverslips and the cells were allowed to settle and attach for 30 min at room temperature. The monolayers were washed, fixed and stained with TRITC-phalloidin as outlined in section 2.8.1.

An *in vitro* phagocytosis experiment was carried out with monolayers from uninjected larvae. The monolayers were prepared as just described and incubated with FITC-*E. coli* under the same conditions and parameters as with *M. sexta* haemocytes (40 µl of 4 x 10^6 bacteria; 2 h at 27 °C incubation). Again, these monolayers were fixed and stained with TRITC-phalloidin

2.19 Phagocytosis in the presence of sodium azide

Sodium azide (NaN_3) was found to prevent phagocytosis by *M. sexta* haemocytes without affecting their viability (as assessed by trypan blue staining). Therefore, this reagent was used to investigate the attachment phase of phagocytosis by haemocytes. Initially, a dose response assay was performed to determine a suitable dose that inhibited phagocytosis (concentrations assayed: 3 µM – 30mM).

Monolayers were incubated in the presence of 3.1 mM NaN_3 for 15 min at room temperature before the addition of FITC-labelled *E. coli* (as in section 2.16.2). Haemocytes were incubated with the bacteria for 2 h at 27°C and were then fixed and prepared for confocal microscopy by TRITC-phalloidin staining. The numbers of bacteria that attached to individual haemocytes (without the confusion of phagocytosed bacteria) were then counted.

2.20 SDS-PAGE and protein staining

In section 2.11, monolayers were produced from *B. bassiana* 304-infected or larvae injected with laminarin at different time points post-treatment. In conjunction with this experiment, the haemolymph samples from the treated larvae were also electrophoresed by SDS-PAGE.

Haemolymph proteins were separated under denaturing conditions by SDS-PAGE using a 10% polyacrylamide gel (according to Laemmli, 1970). Electrophoresis was performed using the Biorad Mini-Protean-II gel apparatus. Whole haemolymph was mixed with loading buffer, boiled for 3 min and centrifuged at 10,000 g for 1 min. Ten µl of supernatant was then loaded onto the gel and electrophoresed at 150 V. For determination of molecular mass, a series of protein standards (6.5-175 kDa, New England Biolabs) was run alongside the haemolymph proteins. The proteins were stained overnight in a solution of 0.1% (w/v) Coomassie brilliant blue with shaking and the gel was destained in a solution of 10% methanol: 7% acetic acid.

Chapter 3

Hyperphagocytic insect blood cells

3.1 Introduction

Phagocytosis is a key component in the immune systems of vertebrates and invertebrates and is considered to be the ultimate mechanism for the disposal of foreign material by the host (Van Oss, 1986). Its importance is exemplified in the mammalian immune system, in which professional phagocytes are dedicated to the ingestion and removal of foreign material (Rabinovitch, 1995). Such specialist cell types have not previously been reported in any insect.

Phagocytosis and nodule formation are important cellular immune responses to infection in insects (Gillespie *et al.*, 1997). Because there is currently intense interest in insect immune responses as simple models of mammalian innate immunity (De Gregorio *et al.*, 2001; Hoffmann and Reichardt, 2002; Irving *et al.*, 2001; Khush *et al.*, 2001; Tzou *et al.*, 2002) and because host immune responses may limit the effectiveness of microbial and parasitoid-based biological control methods directed against insect pests (Alleyne and Wiedenmann, 2001; Connick *et al.*, 2001; Dean *et al.*, 2002; Gaugler *et al.*, 1997; Sagarra *et al.*, 2000; Van Sambeek and Wiesner, 1999; Wang *et al.*, 1994), it is important to know which defensive cell types are involved. Hitherto, two haemocyte classes, the granular cells and the plasmatocytes have been considered to be the main defensive cell types in insect immunity, responsible for both phagocytosis and nodule formation (Gupta, 1991).

Here I report the existence in larvae of the widely studied model lepidopteran, *Manduca sexta*, of a haemocyte type that displays all the characteristics of a professional phagocyte. Despite the fact that these hyperphagocytic (HP) cells are rare in the haemolymph, they are responsible for the majority of phagocytosis when bacteria are experimentally injected into *M. sexta*. HP cells display phagocytosis in extreme degree, each cell capable of becoming engorged with several hundreds of *Escherichia coli* cells during an infection. Moreover,

evidence is provided that supports the idea that HP cells play an important role in nodule formation initiation, revealing these two characteristic cellular immune defences of insects to be closely linked.

3.2 Results

3.2.1 Phagocytosis by haemocytes *in vivo*, following injection of bacteria

3.2.1.1 Plasmatocyte and granular cell frequency in injected and healthy larvae

Haemocytes were examined in monolayers from *Manduca sexta* larvae that had been injected 4 h previously with 5 x 10^7 FITC-labelled *E. coli*. The haemocytes were stained with TRITC-labelled phalloidin, which labels the F-actin cytoskeleton, and the monolayers were then examined by phase contrast or confocal microscopy.

Most adherent haemocytes from experimentally injected larvae could be readily classified visually by phase contrast microscopy (Horohov and Dunn, 1982; Willott *et al.*, 1994) as plasmatocytes (PL, 30.5 ± 4.8%) or granular cells (GR, 60.2 ± 5.9%) (means ± SD, n = 6 larvae, Fig. 1). PL were recognised as phase dark cells that spread on the glass substrate and had relatively unrefractile (non-granular) cytoplasm. GR were refractile (granular) in appearance and had a rounded morphology, adhering to glass by small peripheral filopodia. PL and GR, when attached to a coverslip, measured 20.0 ± 2.2 μm and 8.4 ± 0.6 μm respectively (means ± SD, n = 40 cells) in linear dimension. Most PL and GR adhered singly to the coverslip, but smaller numbers of both PL and GR were also found associated with microaggregates of cells (here defined as 5-20 cells). Such microaggregates are considered to be incipient nodules (Gunnarson and Lackie, 1985; Miller and Stanley, 2001).

Uninjected (healthy) larvae had very similar haemocyte profiles to larvae that were injected 4 h previously with bacteria (Fig. 1). There was no significant difference between the PL and GR frequencies of the two experimental groups of larvae (Mann-Whitney U-test, $P > 0.2$ for both haemocyte types).

3.2.1.2 Quantification of phagocytosis *in vivo*

Phagocytosed FITC-labelled *E. coli* could be easily recognised within haemocytes because (unlike uningested bacteria) their fluorescence was not quenched by trypan blue. Haemocyte monolayers from larvae injected with FITC-labelled *E. coli* were assessed for phagocytosis. Most haemocytes in the monolayer (67.3 ± 6.5%, mean ± SD, n = 6 larvae) did not phagocytose any of the injected bacteria. Of the haemocytes that did phagocytose bacteria, most ingested only a small number (PL: 3.06 ± 0.38 bacteria per cell; GR: 1.77 ± 0.15 bacteria per cell; ca. 250 cells counted for each of 6 larvae).

Also observed, however, were a small number of adherent cells that were spectacularly different in appearance. These cells appeared to be completely filled with huge numbers of phagocytosed bacteria (Fig. 2) and are termed hyperphagocytic (HP) cells. HP cells were always present in monolayers from injected larvae but were present at low frequency (i.e. 1.62 ± 0.29 % of adherent haemocytes [mean ± SD n = 7 larvae], Fig. 1).

Confocal optical sections (1.5 μm in depth) were made along the z-axis (vertical dimension) of individual HP cells. Despite the high levels of phagocytosed bacteria, the optical sections enabled an accurate determination of the numbers of bacteria within HP cells which was 271 ± 15.6 bacteria per cell (mean ± SE, n = 45 HP cells from 9 injected larvae). This was massively higher than that found in non-HP cells (the overall mean ± SD for GR and PL was 2.84 ± 0.9 bacteria, mean ± SE, n = 700 cells). Fig. 3 gives a distribution of the number of ingested bacteria into the different haemocytes. Thus, despite the rarity of HP cells, because they contained so many bacteria, the majority of phagocytosed *E. coli* present on the slide (approx. 80 %) were within HP cells.

Without the use of cytoskeletal staining and confocal microscopy, it would have been difficult to identify HP cells. The intense fluorescence from the large numbers of ingested bacteria often obscured the HP cell and, using conventional epifluorescent microscopy, the ingested bacteria appeared simply as clumps of bacteria on the monolayer. These clumps were easily overlooked or dismissed as artefacts. The combination of phalloidin staining and confocal

microscopy revealed these bacterial clumps to be bound by a thin layer of cytoplasm from the HP cell (Fig. 4). In addition, the quenching of extracellular bacteria with trypan blue along with the confocal optical sections of HP cells revealed unequivocally that the bacteria were intracellular.

The optical sections (0.5 μm in depth) enabled vertical reconstructions of HP cells (Fig. 5A and B). Perhaps because HP cells were so full of engulfed bacteria, their ability to spread was impaired which gave them a rounded morphology. I found that adherent HP cells without large numbers of ingested bacteria were able to spread extensively (see next section) when they assumed a flat morphology.

3.2.1.3 Microaggregate association with HP cells

Bacteria-filled HP cells were often found associated with each other or in haemocyte microaggregates (Fig. 6A and B). Most microaggregates (71.3 ± 17.4%, mean ± SD, n = 16 larvae) were associated with HP cells (range 1-15 HP cells per microaggregates), revealing a strong link between the two processes. Most observed HP cells, however, were solitary and found outside microaggregates, particularly at short times (0.5-2 h) after the injection of bacteria (Fig. 6C). As early as 30 min post-injection (see below), most microaggregates observed in the monolayer were associated with HP cells (Fig. 6D).

3.2.1.4 *In vivo* time course

The frequency of HP cells and their association with microaggregates was assessed over a 24 h period following the injection of FITC-labelled *E. coli*. The number of free bacteria in the plasma decreased rapidly after injection (Fig. 7C) and this coincided with the uptake of bacteria into HP cell types (Fig. 7A). At 30 min post-injection, the general level of phagocytosis by all haemocytes was low and there were few microaggregates. At this time, however, a small proportion (0.7 ± 0.2%, mean ± SD from 4 larvae) of the haemocytes (presumably HP cells) were found associated with huge numbers of bacteria; these cells were invariably associated with the microaggregates (Fig. 6D). By 24 h post-injection, HP cells

were less frequent suggesting they had been removed from circulation, possibly involved in nodule formation. Microaggregates were observed at all time points as shown in Fig. 7B and were invariably associated with HP cells at each time point.

3.2.2 Phagocytosis *in vitro*

3.2.2.1 Haemocytes from healthy larvae

It was shown that HP cells are present in *M. sexta* haemolymph before exposure of the insect to the bacteria, and that these cells are characterised by a distinctive morphology and extreme spreading on glass. When monolayers were prepared from untreated larvae by the same methods as above, most cells were again clearly recognisable as PL or GR (Fig. 1), but a small proportion of the cells (0.86 ± 0.16 %, mean ± S.D., n = 2000 cells counted for each of 6 larvae, Fig. 1) displayed a novel morphology characterised by extreme spreading with abundant peripheral actin-containing adherent spikes. Staining of these novel haemocytes (Fig. 8A and B) revealed that they possessed a well spread central nucleus with a prominent nucleolus and unlike most spread plasmatocytes, had few stress fibres in the cell body. Scanning electron microscopy supported the confocal images that these cells were very large and thin when spread on a glass slide (Fig. 8C).

3.2.2.2 Quantification of phagocytosis *in vitro*

Upon incubating haemocyte monolayers from healthy larvae with FITC-labelled *E. coli* for 2 h at 27°C, these novel cell types ingested very large numbers of bacteria, revealing themselves as HP cells (Fig. 9). Although the non-HP cells had the ability to phagocytose *E.* coli *in vitro*, the number of bacteria engulfed by these cells was small and many cells did not phagocytose at all. The frequency of HP cells observed from uninjected larvae was low and similar to that seen in the *in vivo* assay with injected insects (Fig. 1). The number of bacteria phagocytosed by these spread HP cells was 70.2 ± 22.7 bacteria per cell and that phagocytosed by other haemocyte types was 2.64 ± 2.54 per cell (means ± SD, n = 170 non-HP cells, n = 40 HP cells, Fig. 10A). The difference in phagocytic activity displayed by HP cells and non-HP cell types *in vitro* was highly significant (P = 0.000, t-test).

Monolayers from healthy (non-injected) larvae at different developmental stages (final-day 3rd instar, mid-4th instar, final-day 4th instar, wandering stage) except the pupal stage, all possessed HP cells with novel morphology and high phagocytic activity as described above.

3.2.2.3 Haemocyte dimensions

The morphology of HP cells (without bacteria), as described, was unusual as they spread extensively on the glass surface and therefore appeared significantly larger than the other haemocyte types (Fig. 11A). Vertical profiles of individual haemocytes (using confocal optical sections) allowed an accurate comparison of thickness between the haemocyte types (Fig. 11B) and revealed that HP cells (without ingested bacteria) were, as expected, much thinner than the other cell types. These data (of haemocyte diameter *vs.* thickness) further permitted an estimate of the overall 3-dimensional shape of the haemocyte types; GR and HP cell engorged with bacteria are rounded cells; PL and HP cells without bacteria are flat cells with a large diameter. Although conventional PL are characterised by their spreading ability, HP cells spread more extensively and spread HP cells were significantly thinner with a significantly larger diameter than conventional PL ($P < 0.01$, Mann-Whitney U-test, $n = 7$ for thickness, $n = 40$ for diameter).

3.2.2.4 Long-term phagocytosis by HP cells *in vitro*

Monolayers were examined after 18 h incubation with FITC-labelled *E. coli* and compared with monolayers exposed to the bacteria for only 2 h. The morphology of the spread HP cells changed *in vitro* as they phagocytosed increasing numbers of bacteria, becoming more rounded and eventually indistinguishable from those that had done so *in vivo*. The number of bacteria engulfed by these HP cells increased massively compared with the HP cells in the 2 h incubation whereas the levels of phagocytosis by PL and GR remained low (Fig. 10B). Rounded HP cells full of bacteria were also observed in the supernatant above the monolayer, suggesting the change in morphology reduced their ability to attach to the coverslip.

3.2.3 Phagocytosis of particles other than *E. coli*

Hyperphagocytosis by HP cells was not limited to Gram-negative bacteria. Large numbers of the Gram-positive bacterium *Staphylococcus aureus* strain H462 (a strain that is non pathogenic to *M. sexta*; personal communication, Dr. E. Feil, Dept. Biol. & Biochem., University of Bath) were also phagocytosed by HP cells *in vivo* (Fig. 12) and *in vitro* to a much greater level than the other haemocyte types (Table 2). The number of phagocytosed *S. aureus in vivo* was markedly less than that for *E. coli* (i.e. 148 *vs.* 271 ingested bacteria respectively). This reduced hyperphagocytosis was associated with an increase in the amount of spreading by HP cells as they attained a similar morphology (i.e. stellate with large diameter) to HP cells observed in the 2 h *in vitro* experiment with *E. coli*. Although the number of *S. aureus* cells injected was nominally the same as for the experiments *E. coli*, it is not clear whether the difference in the extent of phagocytosis between the two bacterial species was due to the nature of the bacteria or to the bacterial cell density.

Both negatively (carboxyl-derivatised) and positively (amino-derivatised) charged 1 μm beads were engulfed at significantly higher levels *in vitro* by HP cells compared with other cell types ($P = 0.00$, t-test, Fig 13A, B and C). Similar results were obtained *in vivo* from larvae injected with beads 4 h previously (results not shown). Phagocytosis of positively charged beads was significantly greater than that of the negatively charged beads in both HP and non-HP cell types ($P < 0.05$). It is possible however, that the concentration of the beads was not equal and this may have been enough to provoke the difference in the response. Although HP cells engulfed much higher numbers of beads compared to non-HP cell types, the number of HP cells taken up per cell was smaller than was the case for (similar sized) bacteria.

3.2.4 Staining haemocytes with monoclonal antibody MS13

The monoclonal antibody mAb MS13 labels conventional *M. sexta* PL but not GR (Willott *et al.*, 1994). This was confirmed in the present study (Fig. 14A) and additionally it was found that MS13 strongly labels HP cells (Fig. 14B). This antibody is known to recognise a membrane protein that is important in plasmatocyte spreading and this result suggests that HP cells share this particular antigen.

3.2.5 Effect of sodium azide on phagocytosis

Sodium azide (NaN_3) was discovered to be a strong inhibitor of phagocytosis by *M. sexta* haemocytes without affecting their viability (Fig. 15). The effect was dose dependent. Phagocytic activity in both HP and non-HP cells was completely abolished in the presence of 3.1 mM azide or above. However, at this concentration, the HP cells retained the ability to adhere to *E. coli*. HP cells had a much higher affinity for the bacteria compared with non-HP cells (Table 3). The difference between the adhesion of bacteria to HP and non-HP cells was highly significant ($P < 0.000$, t-test). The bacteria that had bound to the spread HP cells (which could be quenched by trypan blue) were commonly observed attached to the filopodial processes of the HP cell (Fig. 16).

3.2.6 Phagocytosis by haemocytes from four other Lepidoptera

Attempts were made to elucidate whether HP cells exist in other insects in addition to *M. sexta*. *In vitro* and *in vivo* phagocytosis experiments were performed with final stage larvae of *Galleria mellonella*, *Spodoptera exigua*, *Spodoptera littoralis* and *Lacanobia oleracea*. The results obtained were inconclusive. The global level of phagocytosis (i.e. by all haemocyte types) in all four Lepidoptera was considerably higher than that observed with *M. sexta* in terms of the proportion of haemocytes phagocytosing and the ingested number of bacteria engulfed per conventional haemocyte. This was quantified for *S. littoralis* revealing that 86.2 ± 7.4% (means ± SD, n = 4 insects) of adherent haemocytes in this insects were involved in phagocytosis *in vivo* (Fig. 17). As shown in section 3.2.1.2, the global level of phagocytosis

seen in *M. sexta in vivo* (32.7 ± 6.5%) was much lower compared with *S. littoralis*. The number of bacteria phagocytosed per conventional haemocyte in *S. littoralis* (13.5 ± 7.15 bacteria, mean ± SD) was also much higher compared with *M. sexta* (2.84 ± 2.62 bacteria, mean ± SD).

In all four insects, no HP cells were found in haemocyte monolayers exposed to bacteria *in vitro*. The same was true for monolayers prepared from insects injected with bacteria except for *S. littoralis*, which possessed haemocytes that were clearly different from the other haemocytes in the monolayer as they contained large numbers of bacteria. Although this was not quantified, these cell types were more frequent than the HP cells seen in *M. sexta*. Thus, in *S. littoralis* at least, specialised phagocytes appeared to be present.

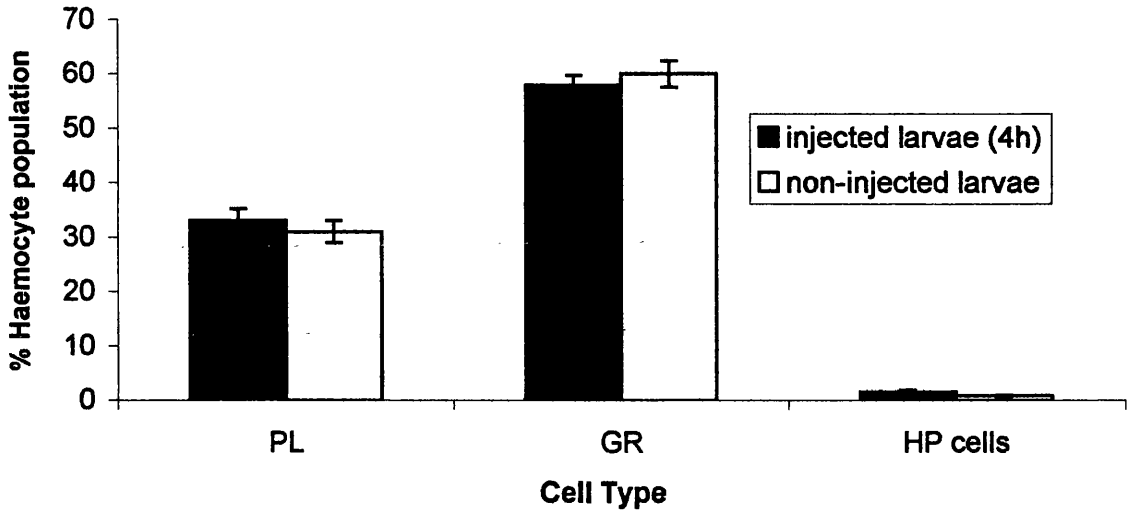
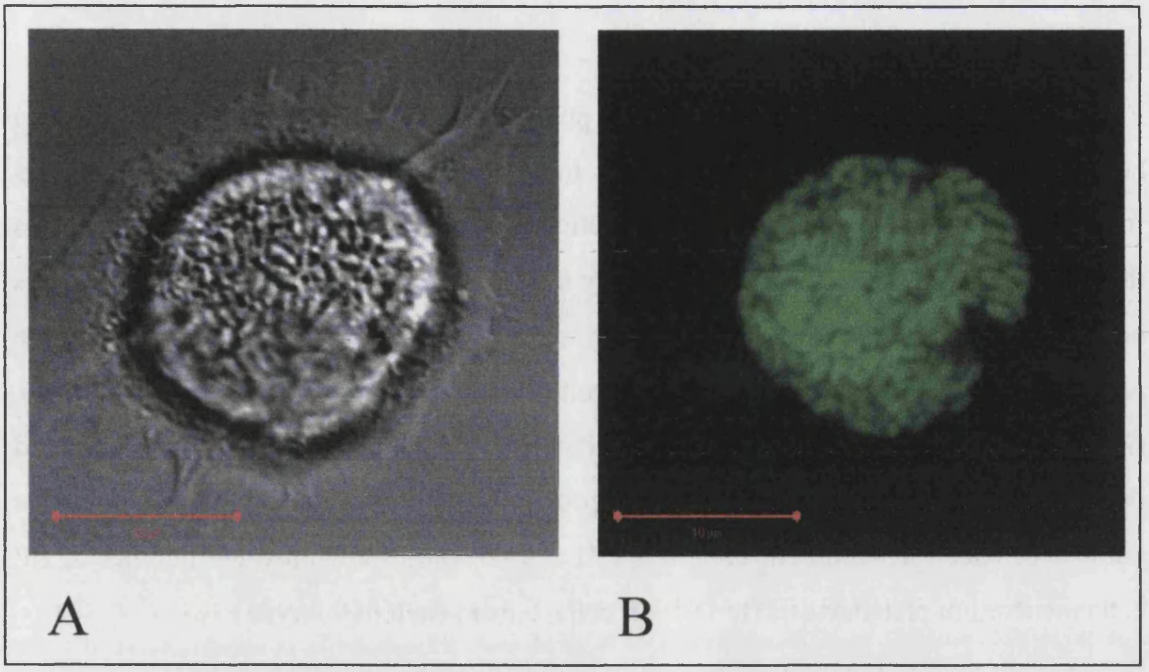
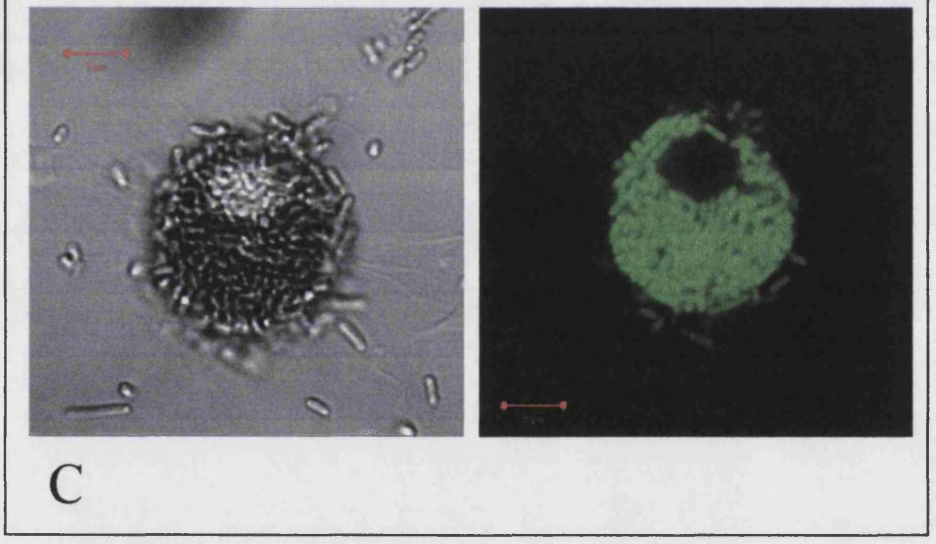


Fig. 1. Frequency of haemocyte types in *Manduca sexta* before and 4 h after an injection of 5 X 10^7 *E. coli*. Three monolayers were made from each of 7 individual larvae and the haemocytes in 6 fields of view were counted per monolayer (approx 1000 cells per larva). The criteria for granular cell (GR), plasmatocyte (PL) and hyperphagocytic (HP) cell morphology are described in the text. Other, minor haemocyte types were included in the total cell count but the specific frequencies of these cell types were not taken. There was no significant change in the frequency of haemocyte types following injection of the bacteria (Mann-Whitney, $p > 0.2$ for each cell type). Bars represent means ± SD ($n = 7$).

Fig. 2. Confocal images of hyperphagocytic (HP) cells from monolayers of *M. sexta* larvae following (4 h) an intra-haemocoelic injection of FITC-labelled *E. coli*. The upper figure shows a transmitted-light (A) and fluorescent (B) image of a HP cell. These two images represent a single optical section through the cell. Many optical sections could be obtained for each cell and were used to measure the number of ingested bacteria (green). The number of ingested bacteria in this particular HP cell was determined to be 261 using these optical sections. The lower figure (C) shows an optical section through another HP cell. The nucleus is clearly visible and surrounded by cytoplasm packed full of bacteria. Bars represent 10 μm and 5 μm for the upper and lower figures respectively.

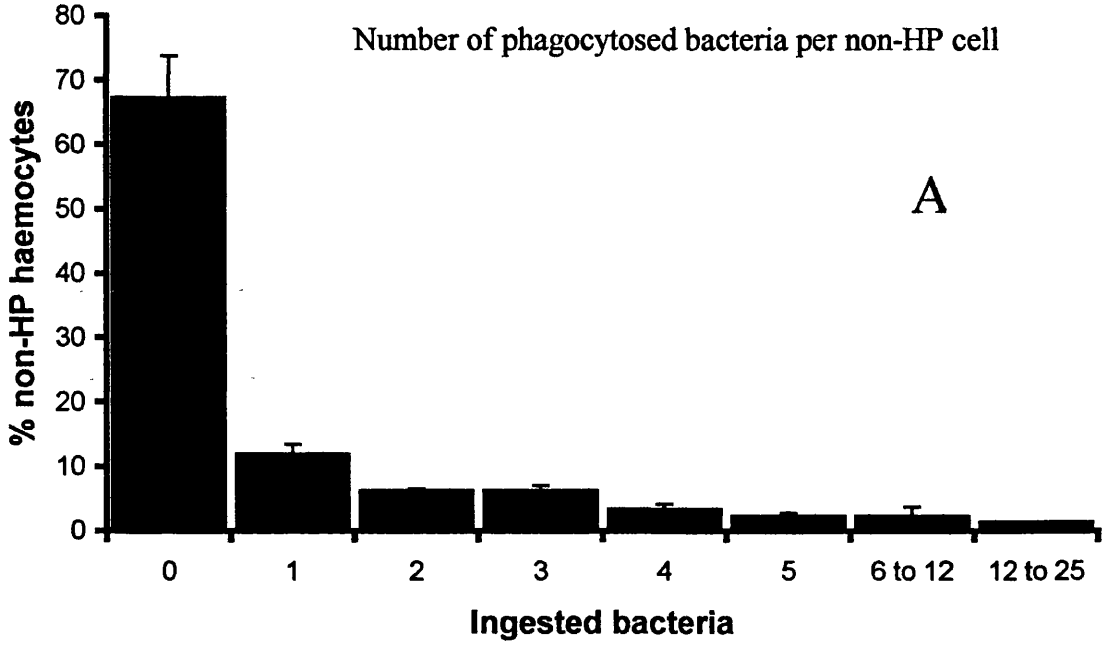


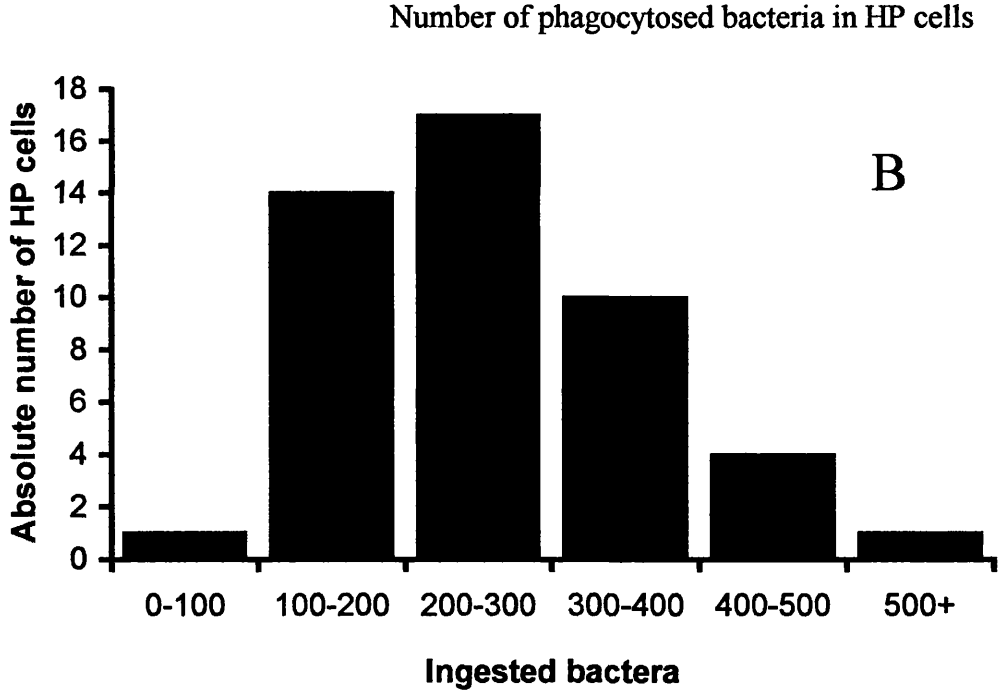
A B

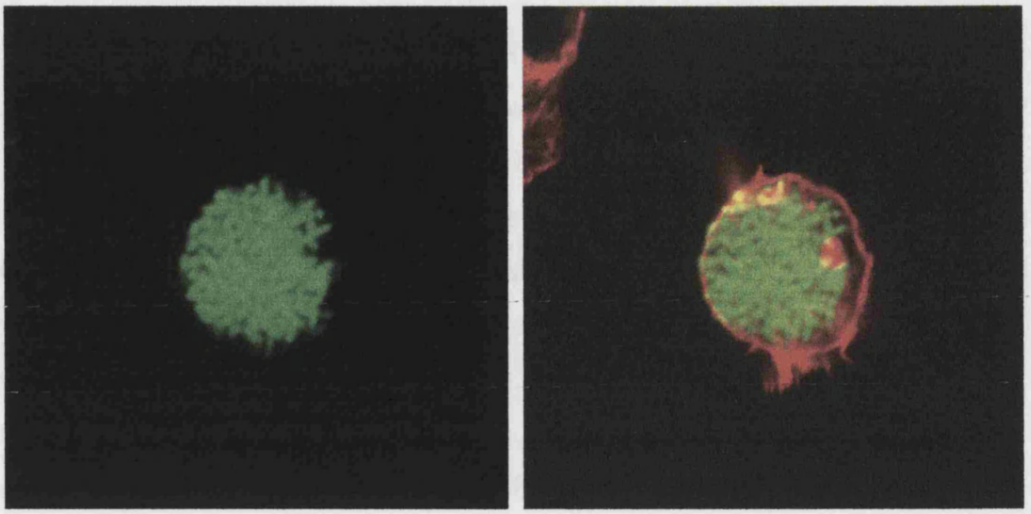


C

Fig. 3. Distribution of the number of phagocytosed bacteria within conventional haemocyte types (non-HP cells, Graph A) and HP cell types (Graph B) in monolayers from larvae following a previous (4 h) injection of FITC-labelled *E. coli*. Graph A shows that most haemocytes have not ingested any bacteria (67%) and where phagocytosis has occurred the level of ingestion is low (mean = 2.84 ± 0.9). Bars show means ± SD of the percentage of haemocytes counted (100 cells counted for each of 7 injected larvae). Graph B shows the levels of ingestion in HP cells. HP cells were selected at random and the number of phagocytosed bacteria was counted as described in the text. The average number of bacteria within HP cells was 271 ± 15.6. Bars show absolute number of HP cells involved in phagocytosis (n = 45 HP cells, 5 from each of 9 larvae).


Number of phagocytosed bacteria per non-HP cell
A
% non-HP haemocytes
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6 to 12
12 to 25
Ingested bacteria


Number of phagocytosed bacteria in HP cells
B
Absolute number of HP cells
18
16
14
12
10
8
6
4
2
0
0-100
100-200
200-300
300-400
400-500
500+
Ingested bactera

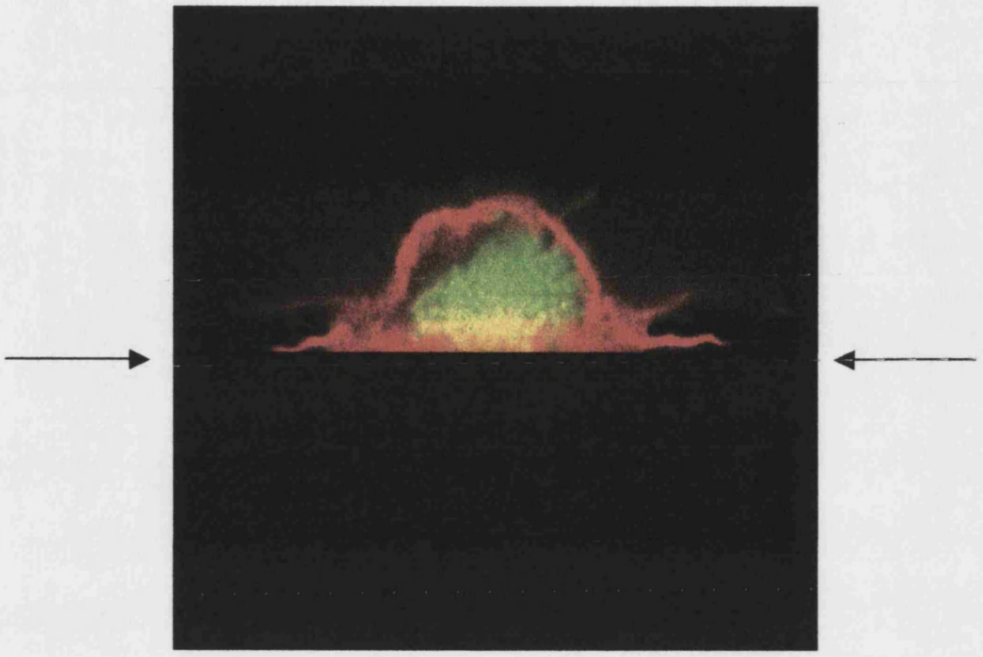


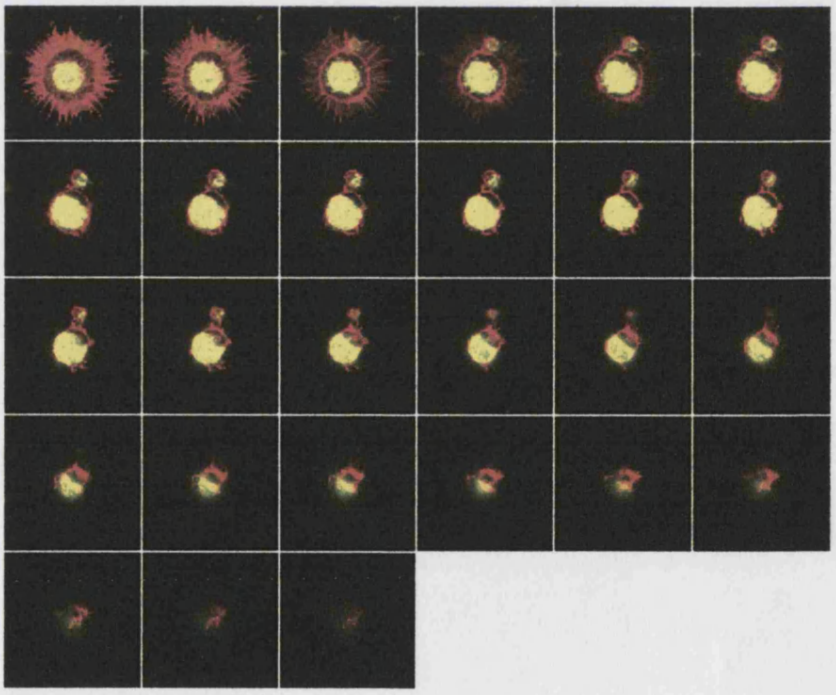
A B

Fig. 4. Cytoskeletal staining with TRITC-labelled phalloidin reveal bacterial clumps to be surrounded by a thin layer of HP cell cytoplasm. Fig. 4A shows an optical confocal section of an FITC-labelled bacterial clump. Fig 4B shows the same bacterial clump combined with the cytoskeletal staining (red) and reveal that the bacteria are within a HP cell.

Fig. 5A. Vertical reconstruction of an HP cell after the phagocytosis of large numbers of FITC-labelled *E. coli* (green). Confocal microscopy was used to make numerous optical sections through the z-axis of the cell. Confocal software enabled these sections to be recombined to create the haemocyte in vertical profile. The arrows represent the level of the glass surface. This method revealed that (a) in agreement with the trypan blue quenching method, the optical sections prove unequivocally that the bacteria associated with HP cells are intracellular (b) much of the HP cell cytoplasm is taken up by the ingested bacteria (c) HP cells (from larvae injected with bacteria) spread little on the glass surface and have a more or less rounded morphology. The thickness of this HP cell is approximately 11 μm. Red = F-actin cytoskeleton (TRITC-labelled phalloidin).

Fig. 5B. Sequence of optical sections of an HP cell after phagocytosis of large numbers of *E. coli*. The haemocyte image in Fig. 5A was constructed using this gallery of optical sections. Each section differs from the previous one by 0.5 μm. At the level of the glass surface (top left), small filopodia can be clearly observed. Bacteria appear yellow due to the co-localisation of the two fluorochromes (FITC-labelled bacteria and TRITC-labelled phalloidin). Note that bacteria are present at almost every section of the cell except in the region of the nucleus (top right of the cell in most section).





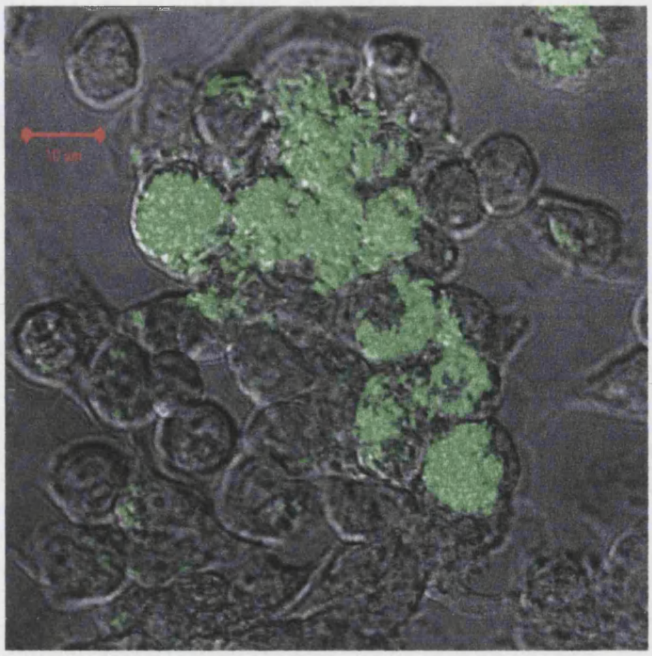


Fig. 6A. A confocal transmitted-light/fluorescent image of a microaggregate (incipient nodule) formed *in vivo* in response an injection of 5 X 10^7 FITC-labelled *E. coli*. Monolayers were made from the injected insects at 4 h post-injection and incubated in 0.2% trypan blue for 20 min to quench the fluorescence from extracellular bacteria. Microaggregates usually contained one or more hyperphagocytic cells (intense green staining). Many of the other haemocytes within the microaggregate had phagocytosed very few bacteria. Bar represents 10 μm.

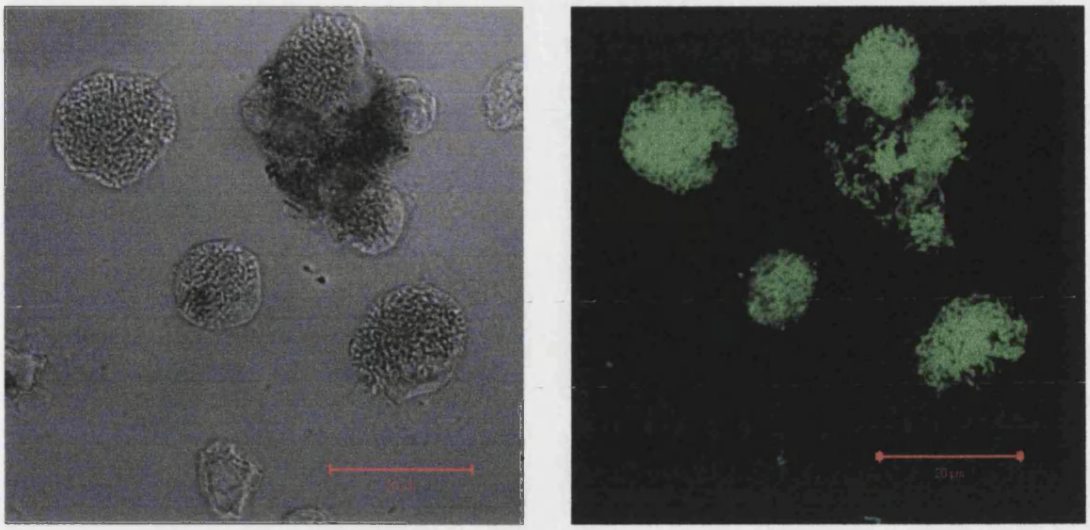


Fig. 6B. Transmitted-light and fluorescence image of HP cell clumps and associated microaggregate (top right). These monolayers were obtained from larvae injected with FITC-labelled *E. coli* as described in the legend to fig. 6A. At least 4 of the cells in this field of view have ingested over 200 bacteria per cell determined using many confocal optical sections. Bar represents 20 μm.

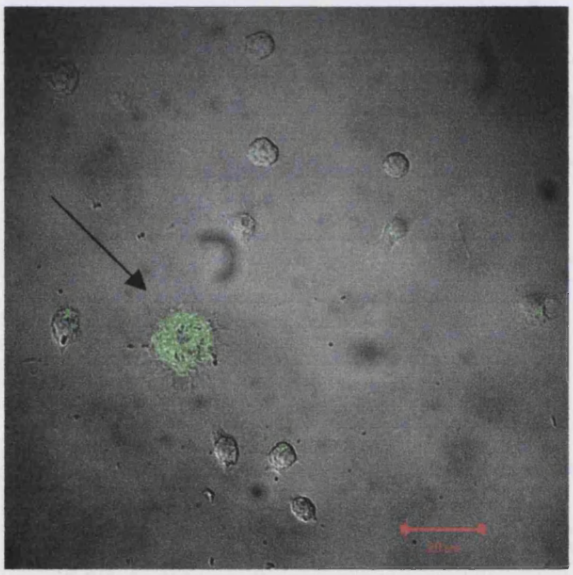


Fig. 6C. Hyperphagocytic cells were commonly found outside microaggregates, particularly at short times following the injection of the FITC-labelled *E. coli*. Most cells in monolayers from injected larvae were not involved in phagocytosis (except HP cells). In this figure, the phagocytic activity between the HP cell (arrow) and the other haemocyte types is clearly different. Bar represents 20 μm.

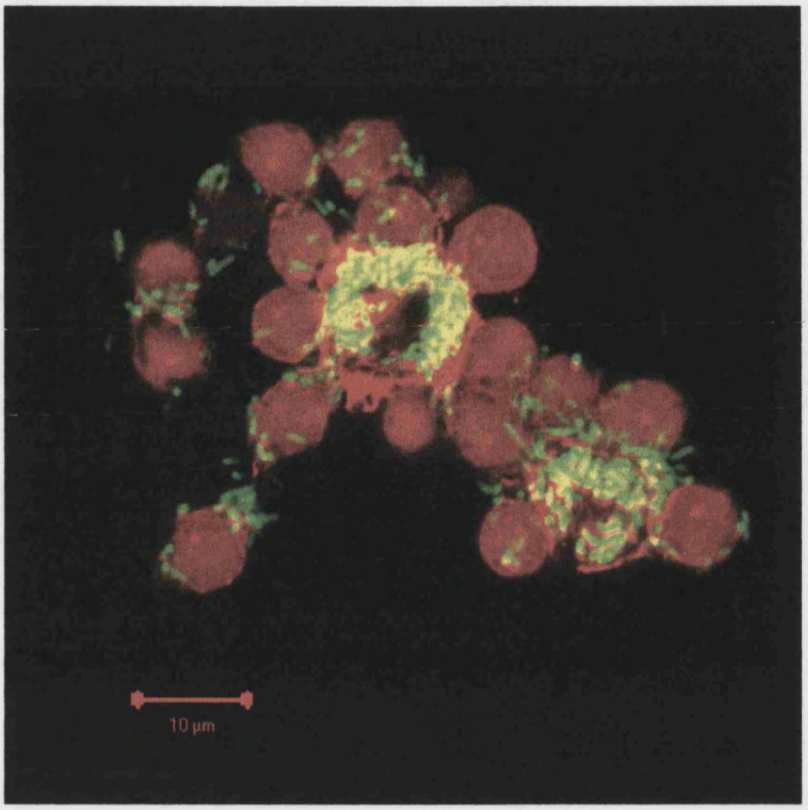
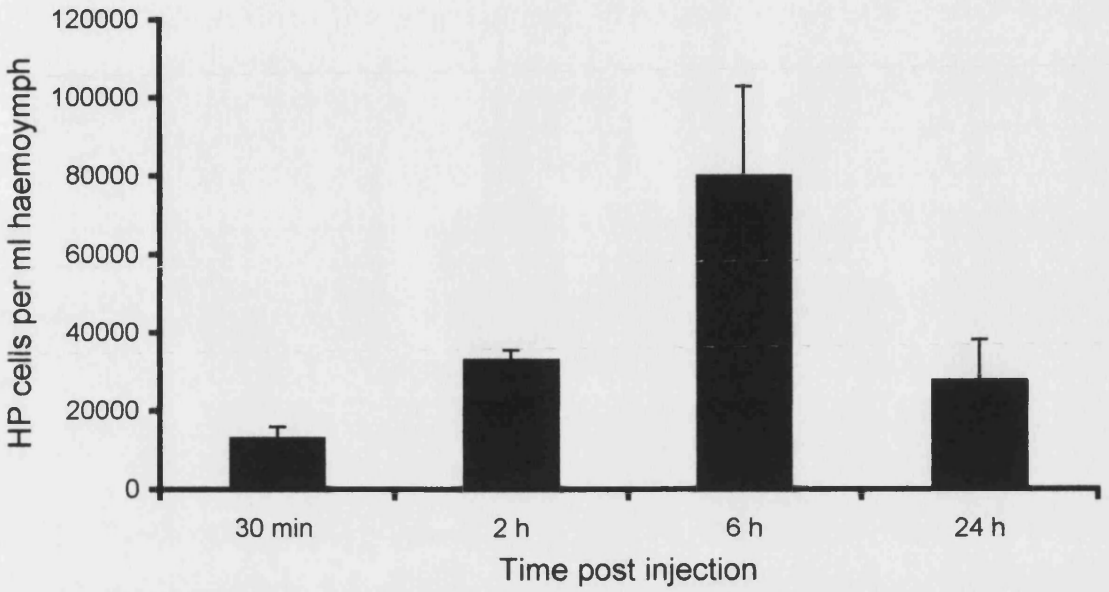
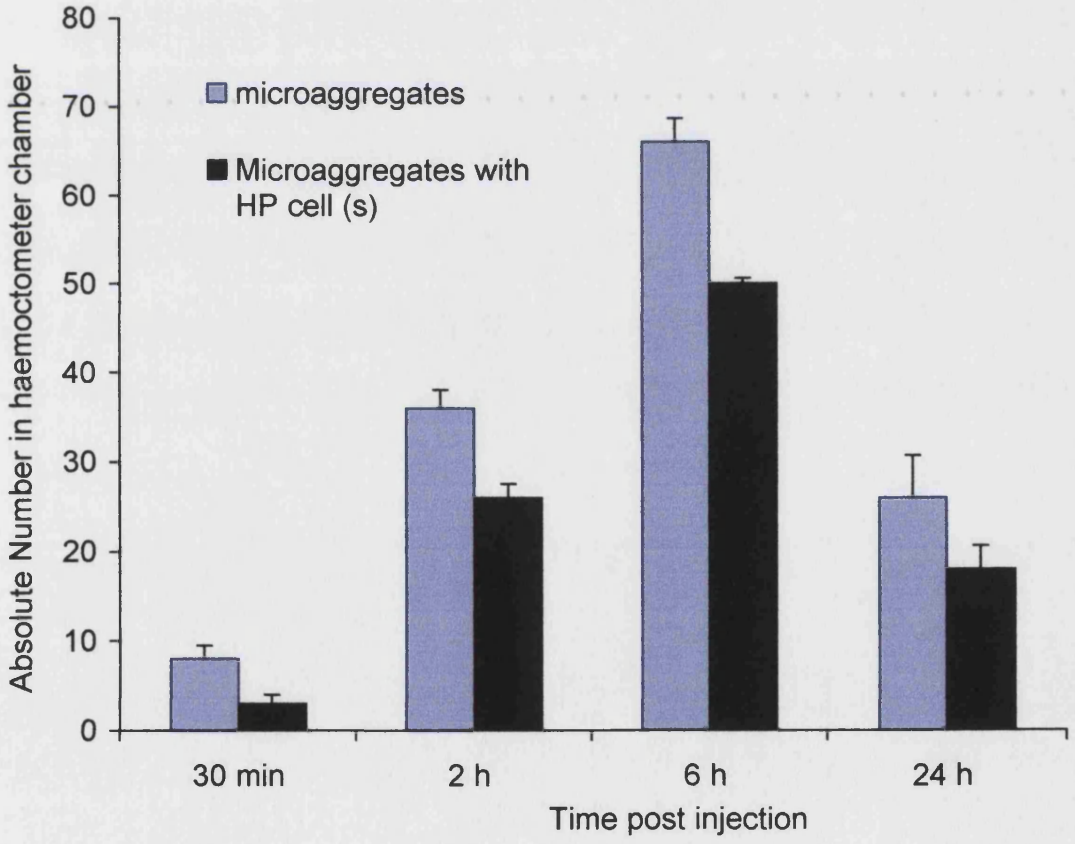


Fig. 6D. TRITC-labelled phalloidin staining of microaggregates of haemocytes in monolayers from larvae injected with FITC-labelled *E. coli* at 30 min post-injection. Most bacteria at this time point have not been phagocytosed but are adhering to the surface of the haemocytes. At the centre of each of the microaggregates is a HP cell with many attached and phagocytosed bacteria. Red = haemocyte cytoskeleton (TRITC-labelled phalloidin), Green = FITC-labelled *E. coli*. Bar represents 10 µm.

Fig. 7A. Number of hyperphagocytic (HP) cells in larvae injected with 5 x 10^7 FITC-labelled *E. coli* at different times post-injection. Larvae were bled and the haemocytes were counted in 9 large squares of a haemocytometer using epifluorescence microscopy. HP cells were identified as haemocytes associated with large numbers of bacteria which were clearly distinguishable as most observed haemocytes were associated with a few or no bacteria. Two haemolymph samples were obtained from each of 4 larvae at each time point. Bars show means ± SD.

Fig. 7B. Time course of microaggregates present in the haemolymph after injection with FITC-labelled E. coli. Two haemolymph samples were taken from each of 4 injected larvae per time point. Microaggregates and HP cells were counted using a haemocytometer. Points represent the means ± SD of microaggregates (blue) and microaggregates associated with one or more HP cell (pink) within 9 large squares of a haemocytometer or 0.9 µl (n = 4 larvae for each point).


120000
100000
80000
60000
40000
20000
0
HP cells per ml haemoymph
30 min
2 h
6 h
24 h
Time post injection


80
70
60
50
40
30
20
10
0
Absolute Number in haemoctometer chamber
microaggregates
Microaggregates with HP cell (s)
30 min
2 h
6 h
24 h
Time post injection

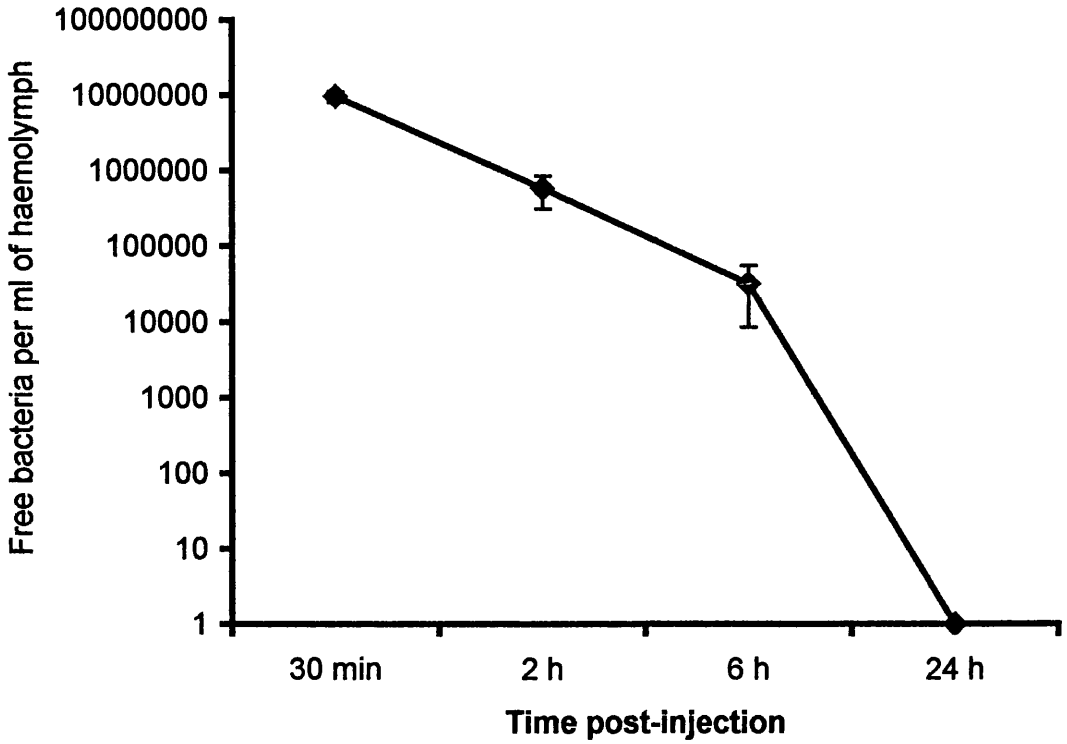


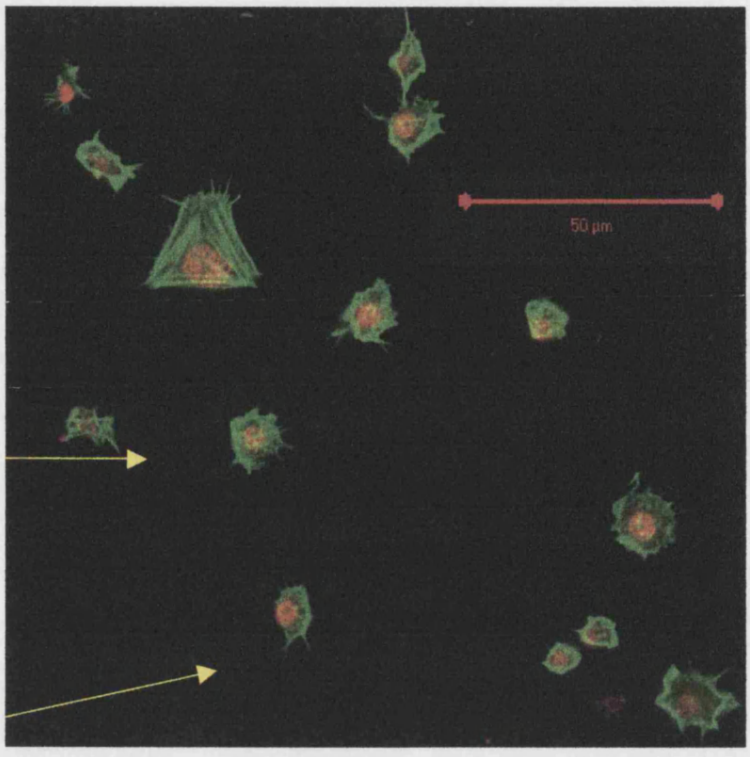
Fig. 7C. Number of free bacteria (*E. coli*) in the haemolymph of injected larvae over the first 24 h post-injection. Larvae were bled directly onto a haemocytometer and the number of bacteria were counted microscopically (at x 200 magnification). Bacteria associated with haemocytes (attached or phagocytosed) were not included. Five larvae were used per time point with 2 haemolymph samples per larva.

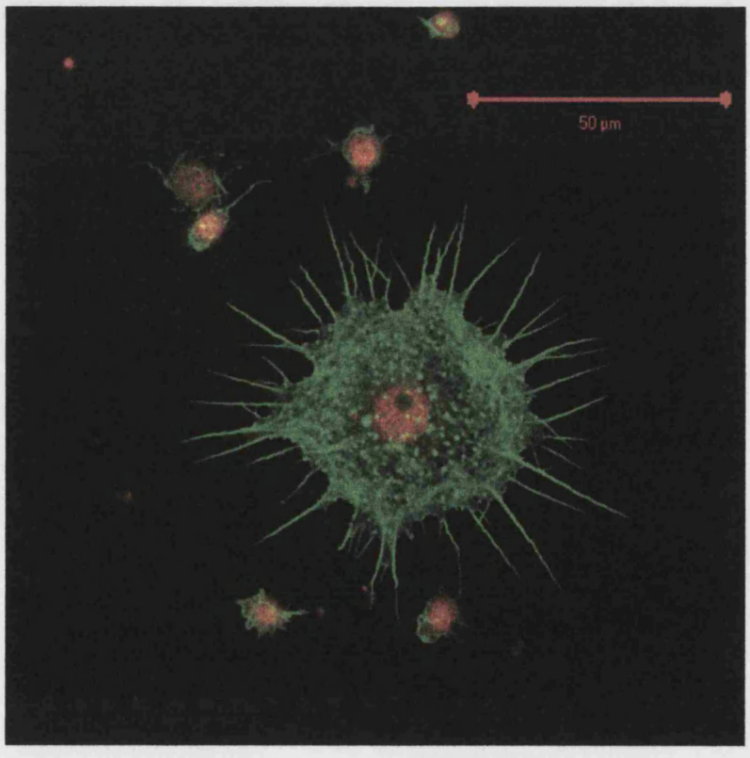
Fig. 8A. Haemocyte monolayers from *M. sexta* typically consisted mainly of granular cells (GR) and plasmatocytes (PL). Cell types were determined by phase-contrast microscopy but were also evident using confocal microscopy when the F-actin cytoskeleton was stained with FITC-labelled phalloidin (green). Red = haemocyte nucleus (propidium iodide). Bar represents 50 μm.

Fig. 8B. Monolayers of *M. sexta* contained a novel haemocyte type. This haemocyte spread extensively on glass, possessed numerous peripheral actin-containing processes and a well-spread central nucleus. Although present in monolayers at low frequency, these novel cell types were consistently present in all untreated larvae. Bar represents 50 μm.

PL

GR





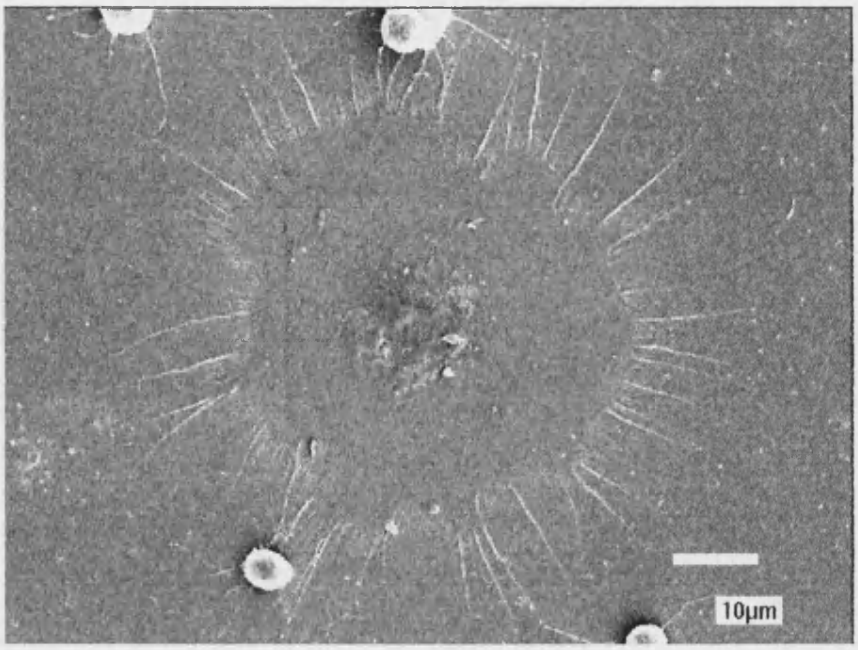
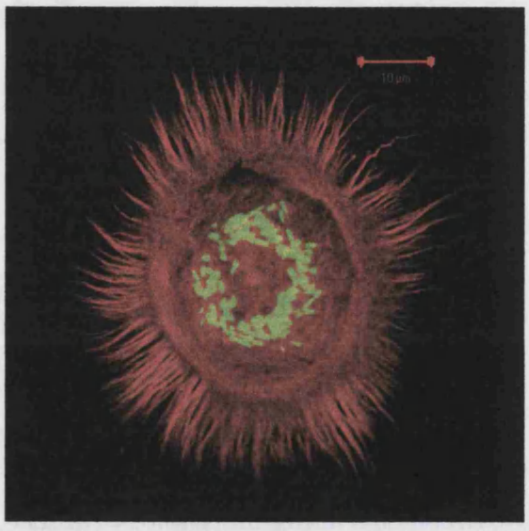


Fig. 8C. Scanning electron micrograph of a novel haemocyte type when spread on a glass surface. These cell types have a very different appearance from other conventional haemocyte types (PL and GR) as they spread extensively (and appeared large and flat) and possess numerous peripheral filopodia.

Fig. 9. Three novel haemocytes (HP cells) ingesting large numbers of bacteria *in vitro*. Monolayers were prepared from healthy larvae were incubated with FITC-labelled *E. coli* for 2 h at room temperature (assay described in the text). The monolayers were then stained with TRITC-labelled phalloidin and viewed using the confocal microscope. Most haemocytes (PL and GR) that displayed phagocytic activity had ingested very few bacteria per cell and many haemocytes had not ingested any bacteria. The novel haemocyte type presented in Fig. 8, with extensive spreading and stellate morphology ingested large numbers of bacteria, revealing themselves to be HP cells. Bar represents 10 μm.



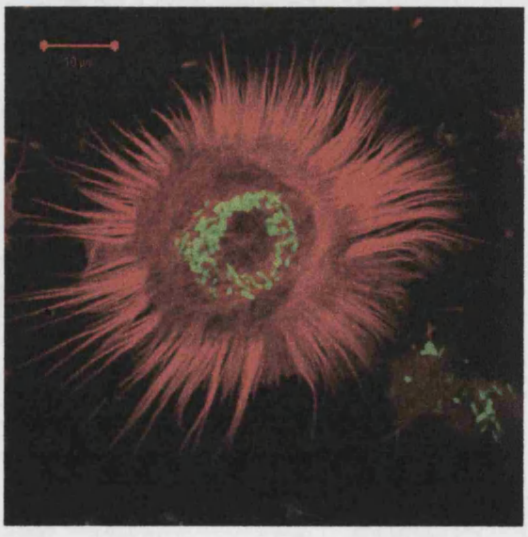
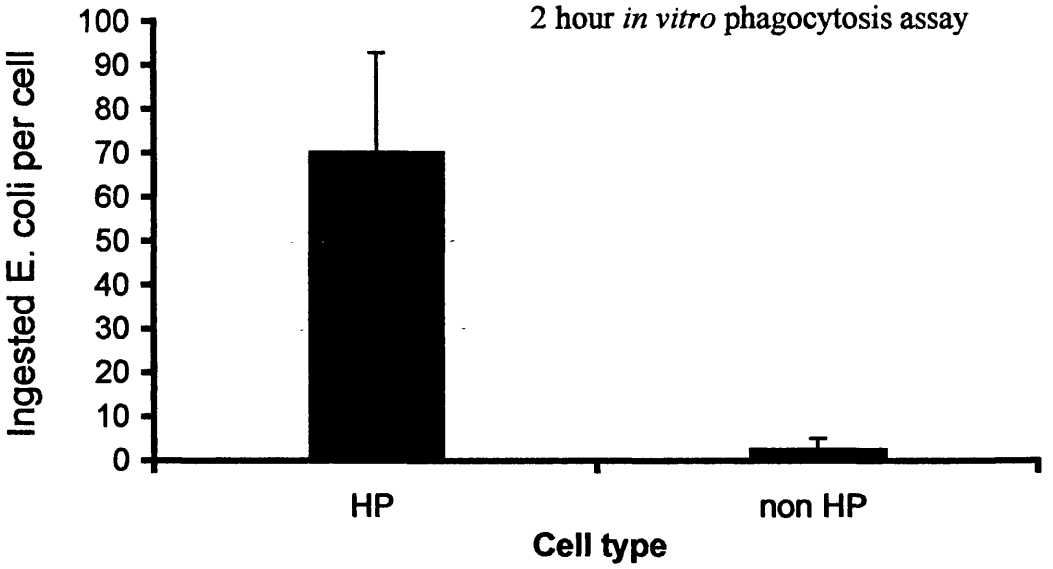


Fig. 10A. Number of phagocytosed bacteria by HP and non-HP cells after 2 h incubation with FITC-labelled *E. coli.* The fluorescence from extracellular bacteria was quenched with trypan blue and monolayers were labelled with TRITC-labelled phalloidin and viewed with confocal microscopy. HP cells, identified by their distinctive morphology when spread on a glass slide were consistently present at low frequency in healthy monolayers. These cell types phagocytosed high levels of bacteria compared with non-HP cells (PL and GR). The difference in phagocytosis between the HP cells and non-HP cells is highly significant (P = 0.000, t-test). Bars represent means ± SD, n = 170 non-HP cells, n = 40 HP cells.

Fig. 10B. Number of phagocytosed bacteria by HP and non-HP cells after 18 h incubation with FITC-labelled *E. coli.* HP cells ingested huge numbers of bacteria compared with conventional haemocytes (PL and GR). HP cells engorged with bacteria after this incubation had rounded up (similar to the HP cells observed in larvae injected with bacteria) and had lost their extreme spreading morphology (observed in the 2 h assay with bacteria, see Fig. 7A). Bars represent means ± SD, n = 15 cells.


2 hour *in vitro* phagocytosis assay
Ingested E. coli per cell
100
90
80
70
60
50
40
30
20
10
0
HP
non HP
Cell type

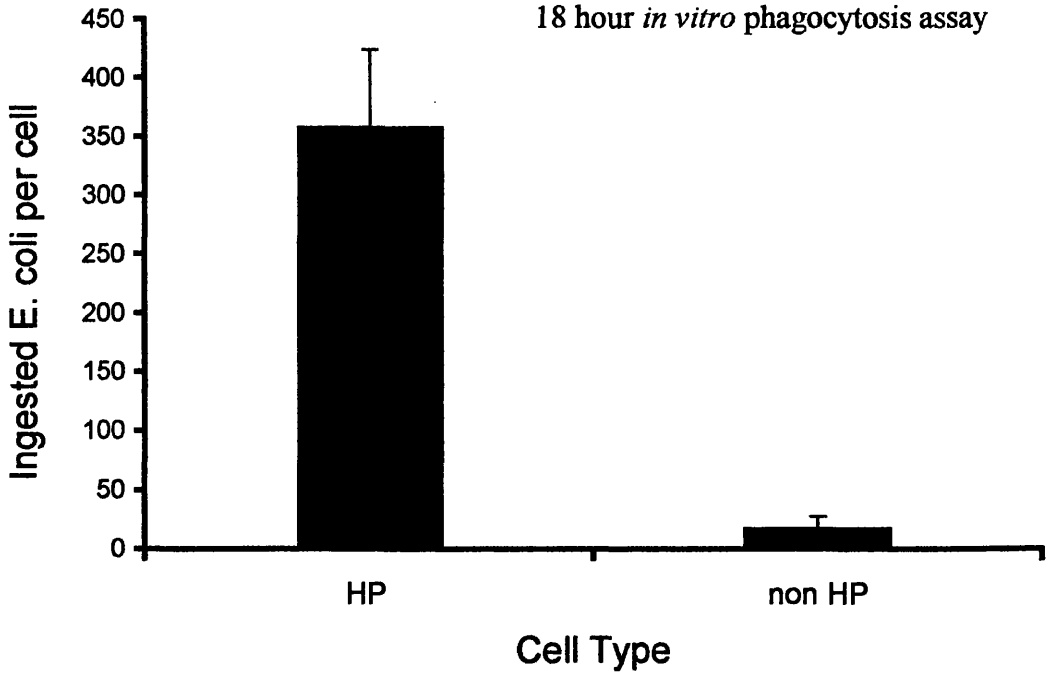
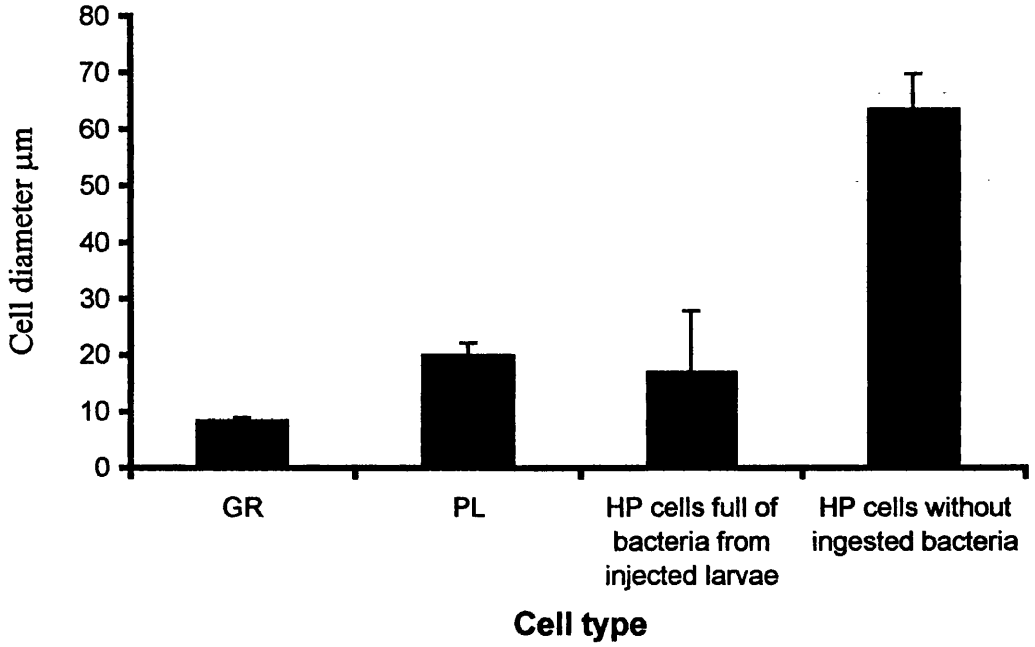

18 hour *in vitro* phagocytosis assay
Ingested E. coli per cell
450
400
350
300
250
200
150
100
50
0
HP
non HP
Cell Type

Fig. 11A. Diameter of haemocyte types when attached to glass. Haemocytes were examined by confocal microscopy after attachment to glass coverslips for 1 h at room temperature. Diameters were determined using confocal software and represent the maximum linear dimension of the cell. HP cells from untreated larvae (non-phagocytosing) appeared large and flat. Plasmatocytes (PL) were commonly observed as spread cells although some were found rounded up. Granular cells (GR) and HP cells engorged with bacteria (from larvae injected with FITC-labelled *E. coli*) had the shortest diameter. Forty cells were measured for each haemocyte type. Bars show mean ± SD.

Fig. 11B. Thickness of haemocyte types when attached to glass. Optical sections allowed accurate measurements of haemocyte type thickness. Granular cells (GR) were very rounded cells that spread very little. Plasmatocytes (PL) were identified with a plasmatocyte antibody (described in section 6.2.4) and although generally well spread, a minority of PL were rounded. Spread HP cells (without bacteria) were very flat and therefore difficult to visualise without cytoskeletal staining. HP cells (full of phagocytosed bacteria) obtained from injected insects were similar to the rounded geometry of GR. Bars represent mean ± SD of cell thickness at the thickest point across the cell (over the nucleus in the case of spread cells), n = 7 randomly selected cells.


Cell diameter µm
80
70
60
50
40
30
20
10
0
GR
PL
HP cells full of bacteria from injected larvae
HP cells without ingested bacteria
Cell type

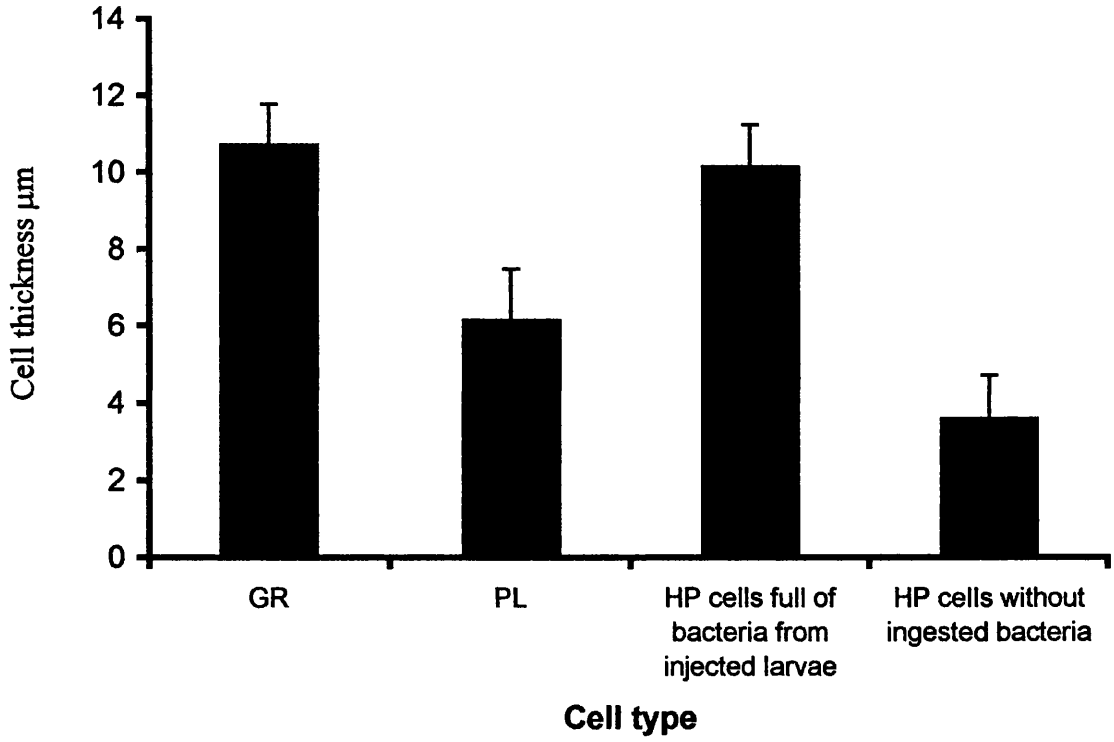
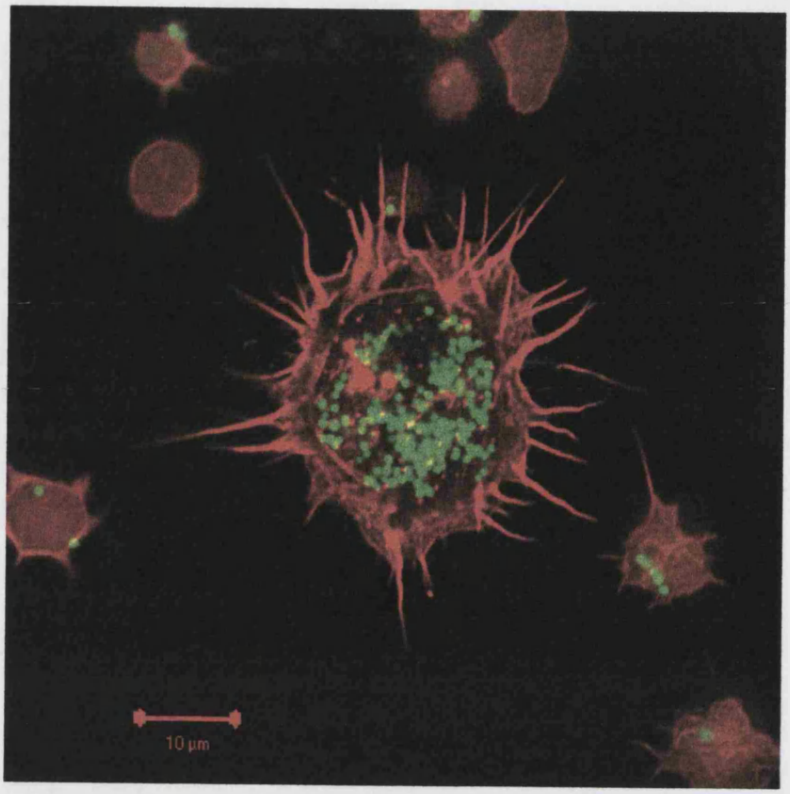
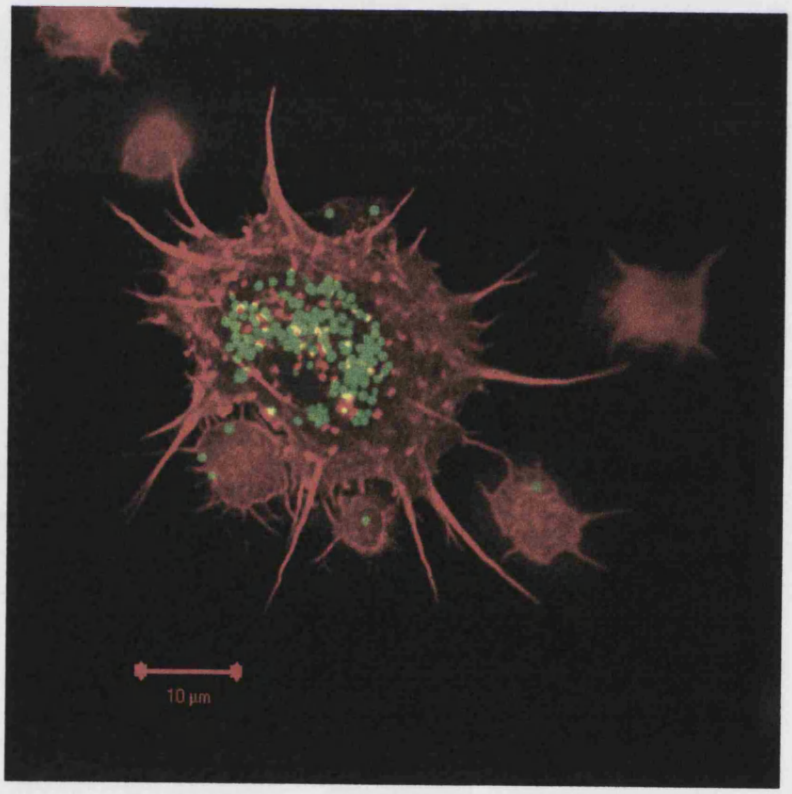

Cell thickness µm
14
12
10
8
6
4
2
0
GR
PL
HP cells full of bacteria from injected larvae
HP cells without ingested bacteria
Cell type

Fig. 12. Phagocytosis of *Staphylococcus aureus* by HP cells following (4 h) an injection of the bacteria. Both figures show *M. sexta* haemocytes in monolayers from larvae injected with 5 x 10^7 *S. aureus*. Most conventional haemocytes (PL and GR) in the monolayer were either non-phagocytosing or had phagocytosed 1-2 bacteria. HP cells had ingested much higher numbers of bacteria. Note the spreading morphology (large diameter and stellate) of the HP cells. Red = TRITC-labelled phalloidin; Green = FITC-labelled *S. aureus*; Bar = 10 μm.


10 µm


10 µm

Cell Type	Number of bacteria phagocytosed	
	Mean	**SD**
HP cell	148.5	51.69 (n = 8)
non-HP cells (e.g. PL and GR)	1.4	1.68 (n = 45)

Table 2. *In vivo* phagocytosis of *Staphylococcus aureus* by haemocytes in experimentally injected larvae. A series of confocal optical slices was used to determine the number of engulfed bacteria within different haemocytes. HP cells were recognised by their distinctive morphology and hyperphagocytic activity (as described in Fig. 12). The level of ingestion of *S. aureus* was considerably lower than that seen with *E. coli in vivo*.

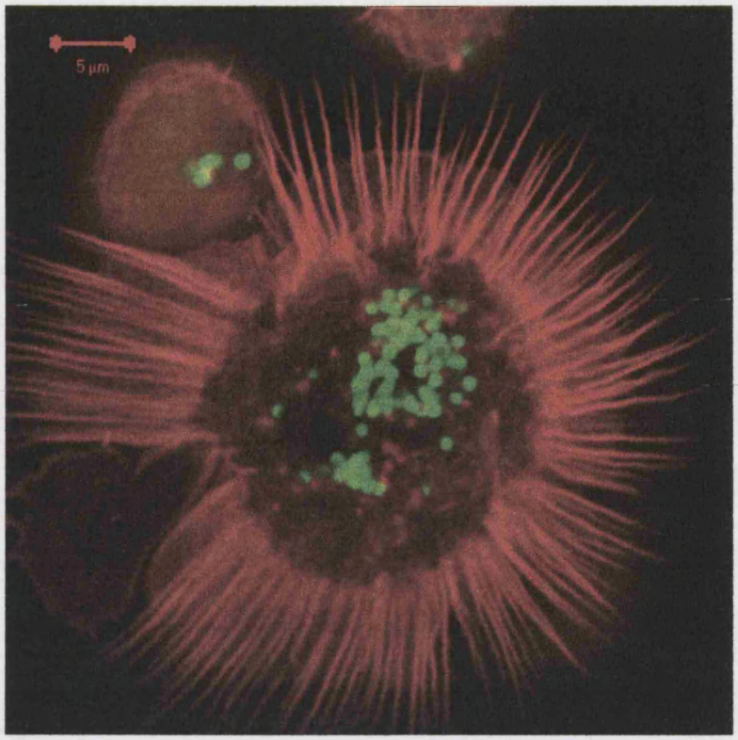
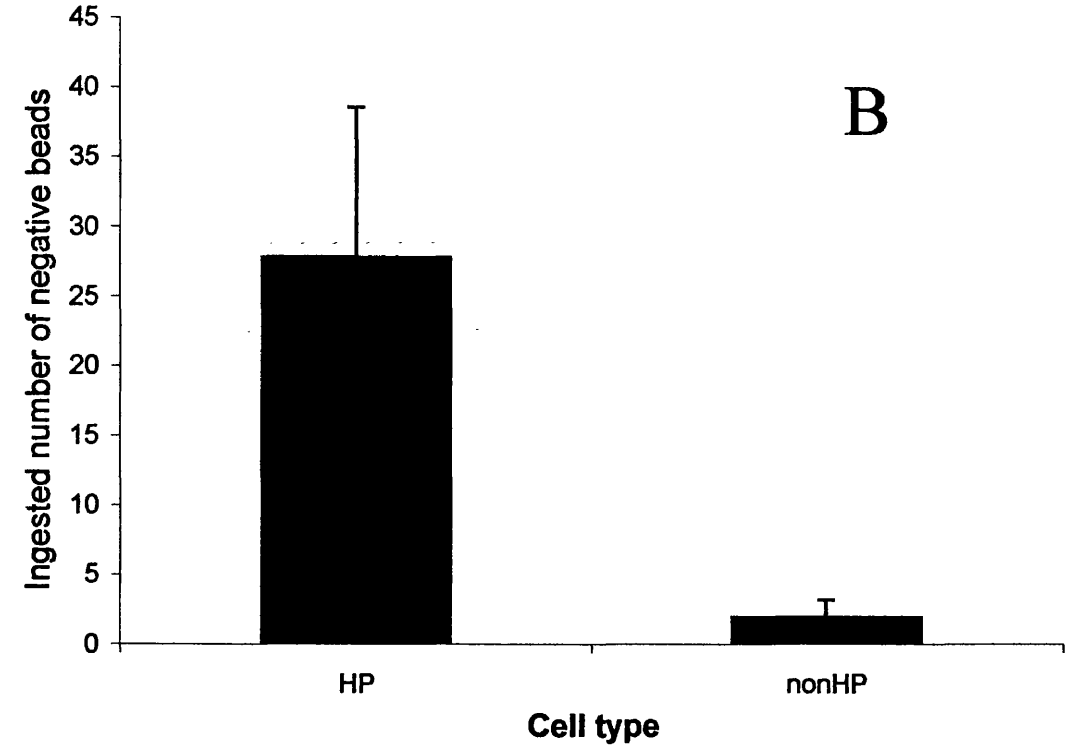
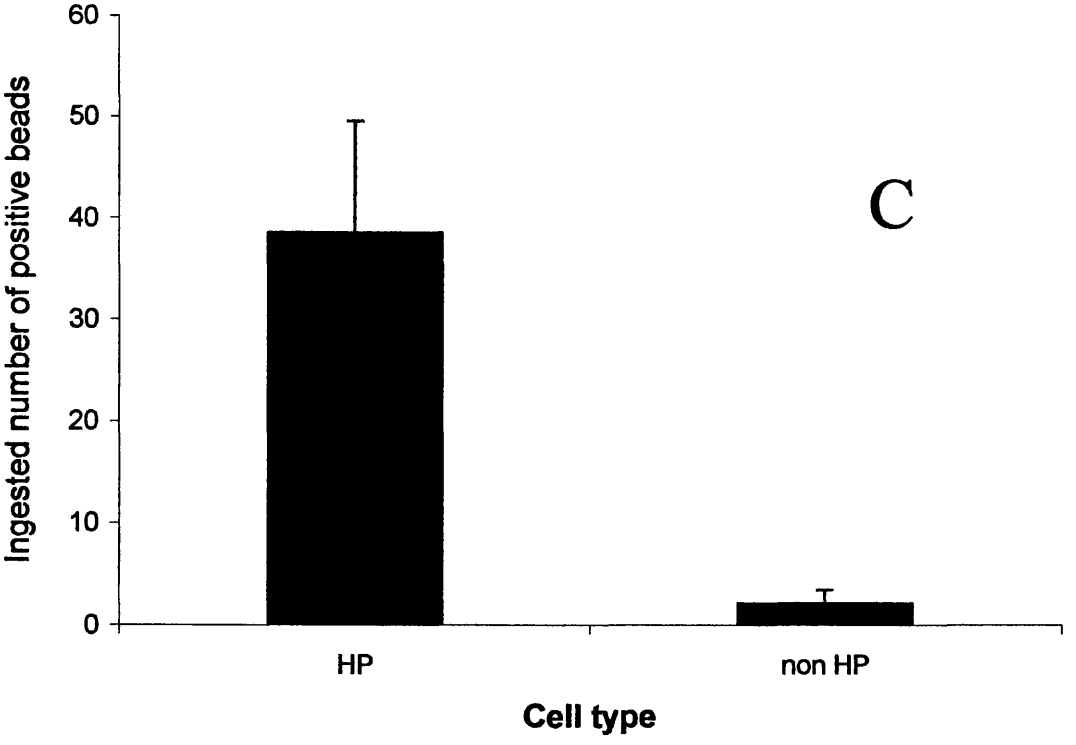


Fig. 13A. Confocal image of an HP cell that has phagocytosed numerous positively-charged microspheres. Monolayers were incubated with FITC-labelled amine-modified 1 µm beads for 2 h as described in the text. HP cells, identified by their distinctive stellate appearance, phagocytosed much higher numbers of beads than non-HP cells. Red = TRITC-labelled phalloidin, Green = 1 µm microspheres. Bar = 5 µm.

Fig. 13B and C. Number of positively and negatively charged beads phagocytosed by HP and non-HP (GR and PL) cells. Monolayers were incubated with the beads (at same concentration for each type) for 2 h at 27°C. Phagocytosis was then assessed only by confocal microscopy as quenching was not possible with trypan blue. For both bead types there was a highly significant difference between phagocytosis by HP cells compared with non-HP cells ($P = 0.000$ for both , t-test). The phagocytosis of positively charged beads was also significantly greater than that of negatively charged beads ($P < 0.05$, t-test). Bars represents means ± SD, n = 376 non-HP cells and 38 HP cells for positive beads; n = 188 non-HP cells and 20 HP cells for negative beads.


B
Ingested number of negative beads
45
40
35
30
25
20
15
10
5
0
HP
nonHP
Cell type


C
Ingested number of positive beads
60
50
40
30
20
10
0
HP
non HP
Cell type

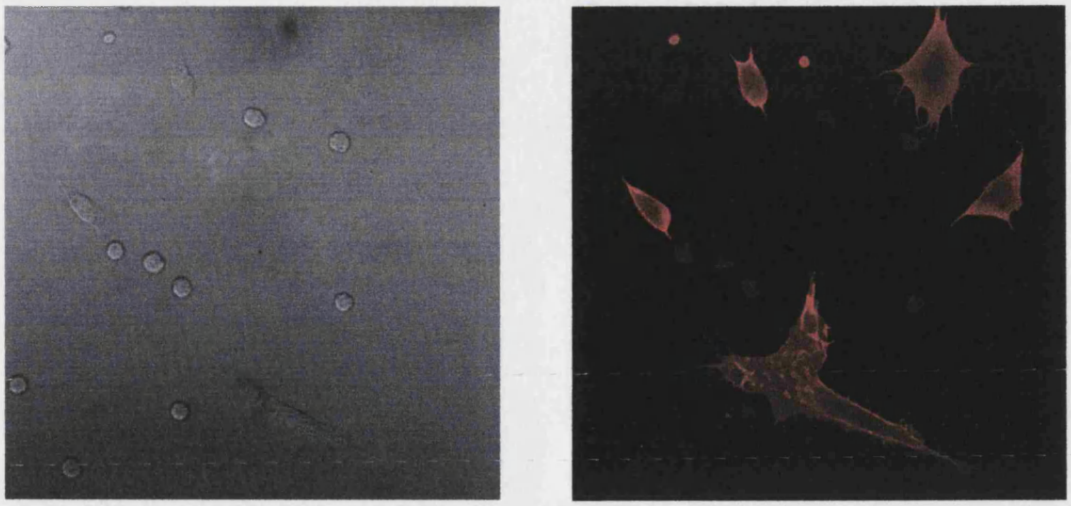


Fig. 14A. Indirect immunofluorescence labelling of plasmatocytes with monoclonal antibody MS13. Both images show the same field of view; left image = transmitted light; right image = fluorescence. Plasmatocytes label strongly with the antibody whereas granular cell do not.

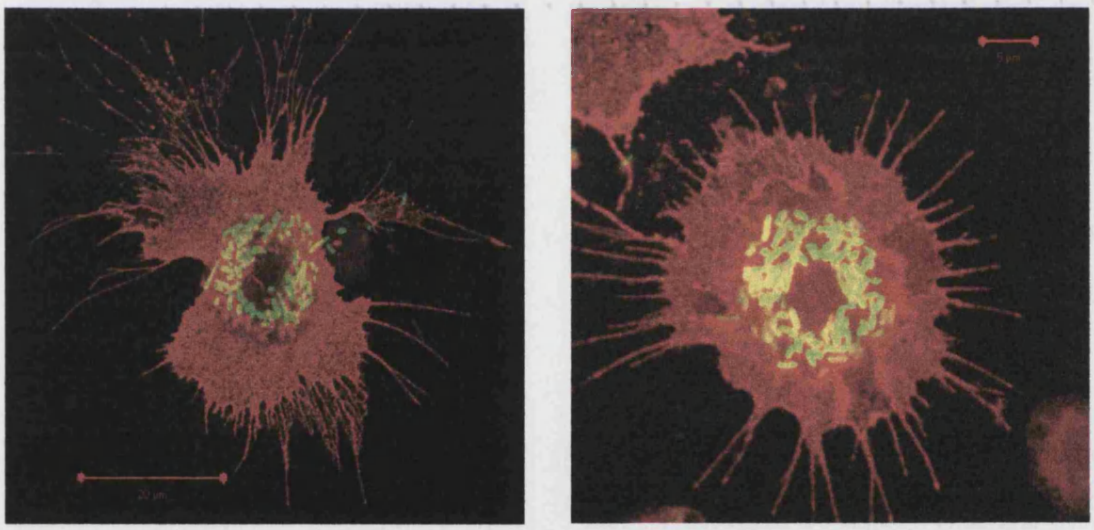


Fig. 14B. Monoclonal antibody MS13 strongly labels HP cells suggesting PL and HP cells share common surface antigens. MS13 recognises a membrane protein and this accounts for the difference in appearance the HP cell compared with phalloidin staining. Red = antibody, green = bacteria. Bar represents 20 μm (left), 5 μm (right).

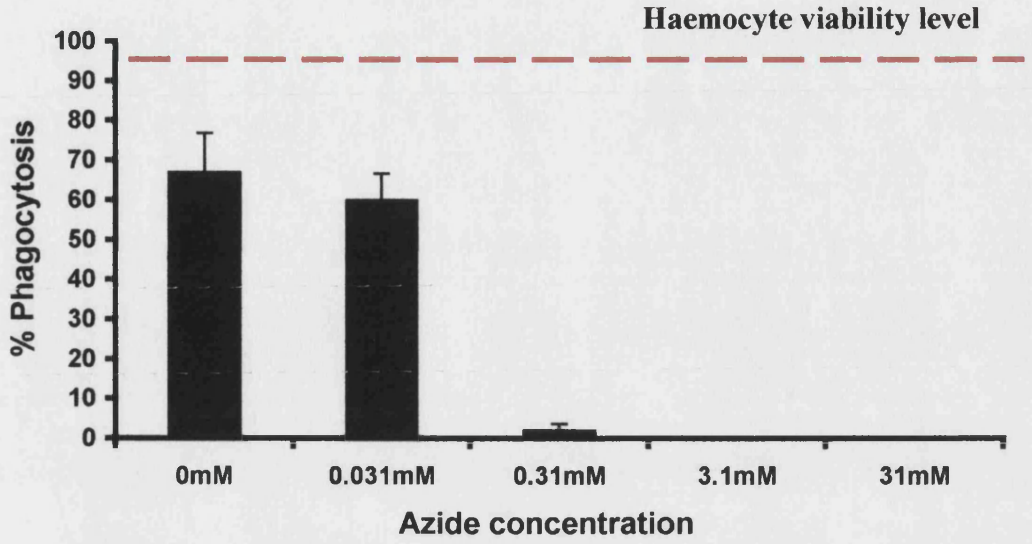


Fig. 15. Effect of sodium azide (NaN_3) on the phagocytosis of *E. coli* by *M. sexta* haemocyte monolayers. Phagocytic activity was completely abolished in the presence of 3.1 mM azide and above. The viability of the haemocytes at all concentrations did not fall below 96% (as assessed by trypan bue). Bars represent means ± SD (n = 10).

Cell type	Number of bacteria attached to the cell	
	MEAN	SD
HP CELLS	77.1	1.22
NON-HP CELLS	3.15	0.41

Table 3. Adhesion of *E. coli* to haemocyte types following the complete inhibition of phagocytosis by sodium azide. Monolayers were exposed to sodium azide and then incubated with bacteria as described in the text. Although all haemocytes were non-phagocytic, there was a huge difference in the affinity of HP cells toward the bacteria compared with other cell types. The azide had no effect on the viability of the haemocytes (remaining above 96% in all fields of view). Figures represent the results from 4 experiments. HP cells: n = 48, non-HP cells: n = 176 cells.

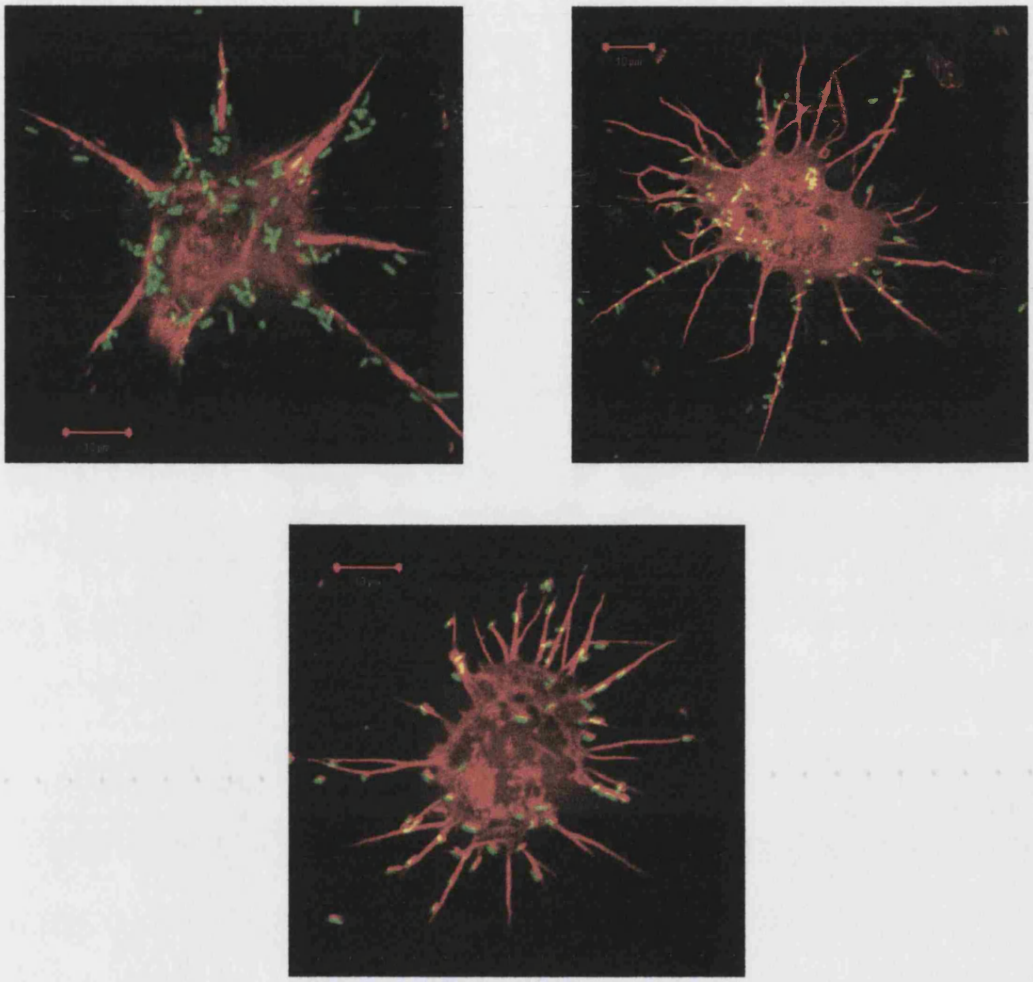


Fig. 16. Adhesion of *E. coli* to HP cells following inhibition of the ingestion phase of phagocytosis with 3.1 mM sodium azide. HP cells were allowed to spread *in vitro* and were then exposed to the azide and bacteria. Attachment of the bacteria by HP cells was high. Red = TRITC-labelled phalloidin, green = bacteria. Bars represent 10 μm.

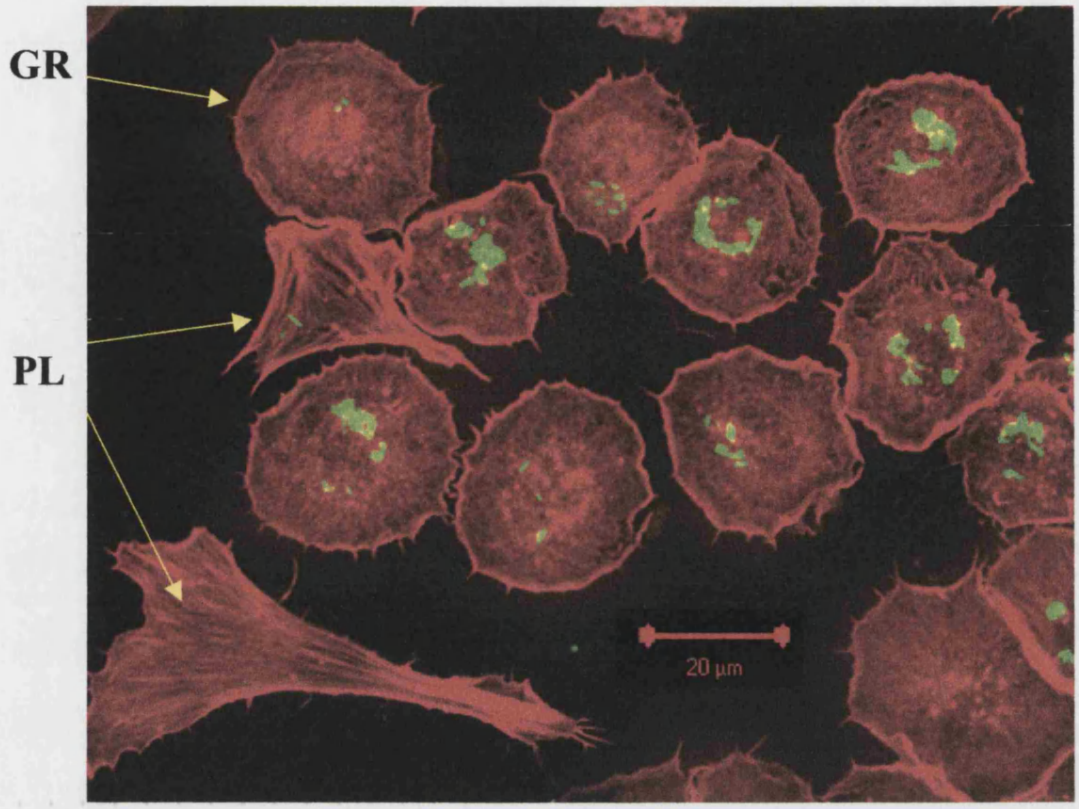


Fig. 17. *In vivo* phagocytosis by haemocytes of *Spodoptera littoralis* following an injection of 2 x 10^7 FITC-labelled *E. coli*. Most haemocytes took part in phagocytosis and the level of phagocytosis by individual haemocytes was relatively high (approx. 13 bacteria per phagocytic haemocyte). Notably, in this insect, the granular cells (GR) were the main phagocytic cell type. Plasmatocytes (PL) were not readily involved in the response. Red = cytoskeleton; Green = bacteria; Bar = 20 µm.

3.3 Discussion

Hyperphagocytic (HP) cells are evidently professional phagocytes. To my knowledge, cells of this type have not previously been observed in insects or any other invertebrate. HP cells have probably escaped previous observation because of (a) their extreme thinness, which makes them almost invisible to conventional phase microscopy (b) their low (but consistent) levels in the haemolymph.

HP cells are likely to play a highly significant role in the immune defences of *M. sexta*, since although they represent around 1% of adherent haemocytes in monolayers, they undertake the majority of phagocytosis (approx. 80%) directed towards bacteria. Assuming the frequency of HP cells in *M. sexta* that adhered to the coverslips reflects their frequency *in vivo*, I can conclude that HP cells constitute the principal phagocytic response to bacterial invaders in this insect. Although both PL and GR do undertake phagocytosis in *M. sexta*, the extent of this response is very small compared to HP cells.

The degree of phagocytosis displayed by HP cells is extreme. I are unaware of any study that reports similar levels of bacterial ingestion by an animal cell. In the greater wax moth, *Galleria mellonella*, Wiesner and Gotz (1993) report high phagocytic activity displayed by a few PL toward 5 μm diameter beads. Most PL in *G. mellonella* ingested 1-2 beads, but the authors also describe plasmatocytes with the ability to engulf up to six beads (each bead about half as large as the haemocyte in their inactive round state). The authors did not attempt to characterise these cells further but commented that such high phagocytic activities were rarely reported.

HP cells are evidently able to phagocytose both Gram-negative and Gram-positive bacteria. Although polystyrene beads with charged surfaces were also engulfed in the experiments, this was to a lesser extent. Since these beads lack microbial pattern molecules, this suggests that phagocytosis by HP cells is promoted by recognition of microbial molecular patterns, such as LPS. Previous studies have shown that LPS promotes phagocytosis by non-specialist insect

haemocytes (Wittwer *et al.*, 1997), and a gene encoding an LPS receptor has recently been shown to be involved in phagocytosis by cells of a *Drosophila* cell line (Ramet *et al.*, 2002).

Bacteria-filled HP cells observed *in vivo* in insects injected with bacteria appeared very different to the well-spread, stellate cells observed *in vitro* in monolayers from uninjected insects. However, four lines of evidence suggest that these cells types are the same. First, (functional similarity), both cell types phagocytosed very large numbers of particles, considerably greater than the other haemocyte types in both the *in vivo* and *in vitro* experiment. Second, when exposed to bacteria over long periods of time *in vitro*, the spreading and stellate cells in the monolayers phagocytosed enormous numbers of bacteria and in doing so rounded up. This rounded morphology was indistinguishable from the HP cells engorged with bacteria *in vivo*. It seems probable that the ingestion of the huge numbers of bacteria impairs the spreading ability of HP cells. Third, their low, but consistent frequency is very similar. Finally, HP cells observed *in vitro* and *in vivo* label strongly with the monoclonal antibody mAb MS13.

MS13 has been reported (Wiegand *et al.*, 2000) to specifically label a 90 kDa haemocyte membrane protein that is required for normal spreading and encapsulation responses in *M. sexta* plasmatocytes. HP cells were labelled strongly by MS13 indicating that the 90 kDa cell surface protein is also present in HP cells. It remains to be seen whether HP cells and PL are derived from a common haemopoietic precursor. It is possible that the 90 kDa cell surface protein recognised by MS13 is not a developmental marker for PL, but is instead simply a marker of actively spreading cells.

HP cells phagocytose enormous numbers of bacteria and it is therefore likely that their cell surface is highly specialised for such recognition. The use of sodium azide in the present study (which prevented the ingestion phase but not the attachment phase of phagocytosis) revealed these cells are indeed highly specialised for recognition of *E. coli*. Other haemocyte types attached to very few bacteria compared with the HP cells, emphasising the functional difference between these cell types. Candidate receptors for such recognition have been well documented in several insect species (Dimopoulos *et al.*, 2000; Werner *et al.*, 2000) and

include, among many, a Gram-negative bacteria-binding protein (Kim *et al.*, 2000) the peptidoglycan recognition protein, PGRP (Ramet *et al.*, 2002) and several putative lectins. Interestingly, a PGRP, identified in the fruit fly *D. melanogaster*, was shown to be specifically involved in haemocyte phagocytosis (Ramet *et al.*, 2002). It cannot be discounted that phagocytic recognition may also occur because of the absence of certain host ligands (self-markers) on the foreign particle (Salt, 1970). If this were so, then HP cells would still require an abundance of 'self-receptors' on their cell surface.

The mechanism of hyperphagocytosis does not appear to require prior opsonization of the phagocytic particles in my experiments. Haemocyte monolayers in the *in vitro* phagocytosis assays were washed free of plasma components before the addition of non-opsonized particles. Although this suggests that opsonisation is not important, Wiesner *et al.* (1996) show that in *G. mellonella* opsonins are released by phagocytosing plasmatocytes into the supernatant when stimulated by foreign agents over a 3 h period. My *in vitro* phagocytosis assay was run for 2 and 18 h, which may have been sufficient time for significant release of opsonins into the above supernatant.

Whether HP cells themselves kill the internalised bacteria remains to be seen. If this were true, HP cells would need to possess an impressive array of lysosomal enzymes and bactericidal metabolites. Another alternative is that HP cells, once sequestered within a nodule, become the target for toxic metabolites such as phenoloxidase products like quinones, from the surrounding haemocytes in the nodule. I observed consistent necrosis of haemocytes in the core of microaggregates and this is well documented in the literature (Rahmet-alla and Rowley, 1989; Ratcliffe and Gagen, 1977).

HP cells (without engulfed bacteria) were identified by their extreme spreading on a glass surface. The reason HP cells spread extensively is unknown but open to speculation. Swanson and Baer (1995) suggest that the high degree of spreading by macrophages *in vitro* is due to a 'frustrated phagocytic' response. If this were true, then HP cells upon contact with the glass surface may be attempting to 'phagocytose' the slide because it is being detected by these cells as foreign. Other haemocyte types (GR and PL) spread to a lesser degree and this could

be due to the reduced phagocytic response of these cells to the glass slide. Interestingly, measurements of cell diameter and thickness of PL, GR and HP cells (which is inversely related for all cell types) imply similar cell volumes suggesting the increased spreading of HP cells compared with PL and GR is not simply due to increased cell size.

The motility of HP cells was not assessed in the present study but is an important discussion point. Macrophages are motile *in vivo* allowing them to infiltrate various internal tissues and phagocytose bacteria within them. As HP cells share common traits with macrophages (i.e. spreading and dedicated phagocytosis) it would not be unexpected to find HP cells as highly motile *in vivo*. Recent work with the entomopathogenic bacterium *Photorhabdus luminescens* reveals that upon infection of *M. sexta*, this bacterium occupies a specific niche under the basal lamina of the insect midgut (Silva *et al.*, 2002). Many pathogens may employ similar strategies in an attempt to avoid the haemocytes. The ability of HP cells to infiltrate localised multiplication sites of potential pathogens would arm the immune system with a useful defensive strategy. Insect haemocytes are highly motile *in vitro* (Takle and Lackie, 1986) and chemoattraction toward bacteria (Kumazawa and Morimoto, 1992, Lopez-Cortez, *et al.*, 1999) and bacterial products *in vitro* (Schneeweiss and Renwrantz, 1993) has also been reported in invertebrates haemocytes.

The high degree of phagocytic activity displayed by HP cells implies substantial plasma membrane internalisation. Upon phagocytosis, each bacterium becomes bound by plasma membrane derived from the cell surface, giving rise to an intracellular phagosome. As a requisite for hyperphagocytosis, HP cells must therefore, possess a highly effective mechanism for the rapid replenishment of plasma membrane to ensure there is no large net reduction in HP cell surface area. Recent studies with macrophages show this to be the case (Cox *et al.*, 2000; Hackam *et al.*, 1998; Lennartz *et al.*, 1997). In these cells, optimal phagocytosis only occurs concomitantly with exocytosis, which acts to deliver endomembranes back to the plasma membrane. It was shown there was even a net increase in macrophage surface area during phagocytosis due to exocytosis (Hackam *et al.*, 1998). In the case of HP cells, an approximate value for membrane internalised during high levels of phagocytic activity may be calculated. Assuming the diameter of HP cells in their native,

rounded state is 10-15 μm (and also assuming the cells are spherical with no membrane ruffles) then the cell surface area is 300-700 μm^2. If the rod-shaped *E. coli* is assumed to be a round-ended cylinder of average size 2.5 μm x 0.7 μm then the total surface area of 350 ingested bacteria would be approximately 1020 μm^2. Therefore, assuming tight bacterial apposition with the phagosomal membrane, the turnover of plasma membrane by HP cells following high phagocytic activity would be between 140-300% of the original cell surface area, implying massive levels of exocytosis by HP cells. Transmission electron microscopy would provide a better idea whether this were true as extensive intracellular pools of endomembranous vesicles would be expected within HP cells, particularly during phagocytosis.

In the present study, efforts to demonstrate the existence of HP cells in *G. mellonella, L. oleracea* and *S. exigua* were largely unsuccessful. Results were negative *in vivo* and *in vitro* but there are several possible reasons why HP cells have not previously been found in these or other insect species:

(a) HP cells in other insects may belong to a fixed haemocyte population, attached to various tissues or contained in specific organs and would therefore not be present in haemocyte monolayers. Studies with *M. sexta* have revealed that haemocytes with hyperphagocytic properties do indeed attach to internal structures such as tracheae (Dr. T. Trenczek, personal communication). Similar fixed phagocytes have been reported in many other invertebrates (Bayne, 1973; Kondo *et al.*, 1998; Matricon-Gondran *et al.*, 1999; Sminia *et al.*, 1979) and described as highly specialized cells designed to sequester large amounts of particulate matter (Johnson, 1987). 'Phagocytic organs' have also been reported in the haemocoel of insects (Jones, 1970; Hoffmann *et al.*, 1974) and crustacea and described as populations of fixed haemocytes, responsible for almost all phagocytosis of injected foreign particles to the exclusion of free haemocytes (Cuénot, 1905; Reade, 1968). This description is similar to the phagocytic response seen with HP and non-HP cells in *M. sexta*. (b) The methods used in this study may not have been optimal for hyper-phagocytosis in the other Lepidopterans. (c) The immune response to *E. coli* may be different depending on insect species. Work with *Galleria* by Wiesner and Gotz (1993) suggest that the wax moth does possess haemocytes with high

phagocytic capacity towards silica beads. These were not seen in the present study. Therefore, the choice of provocator may be an important factor in determining whether HP cells exist in insects other than *M. sexta.* (d) Other insects may use a different immune strategy to combat invaders. The general levels of phagocytosis was much higher in the insects tested compared with *M. sexta.* Most haemocytes *in vivo* were phagocytic and ingested relatively large numbers of bacteria. Thus, some insects may not need HP cells.

The haemocytes of *S. littoralis* haemocytes did display some hyperphagocytic properties *in vivo.* Although not quantified, these cells appeared to be present in higher frequency *in vivo* than was the case with *M. sexta.* However, the general phagocytic response (i.e. by all the haemocytes) to the bacteria in this insect was also very high and clearly different from *M. sexta.*

It is clear that, at least in *M. sexta,* HP cells play an important role in immune defence against bacteria. After injection with FITC-labelled *E. coli,* the numbers of bacteria-filled HP cells increased to a maximum at 6 h post-injection and this is concomitant with an increase in the observed microaggregates and a decrease with free bacteria in the plasma. Horohov and Dunn (1983) carried out a similar time course experiment with *M. sexta,* examining the viable counts of bacteria in the haemolymph at different times post-injection. In their experiment however, the Gram-negative bacterium *Pseudomonas aeruginosa* was used. They report a two-stage process of bacterial clearance: Stage I (0-2 h) in which most bacteria (>90%) were entrapped by nodule formation during the first 30 min after injection; Stage II (2-8 h) in which the nodules and haemocyte clumps had completely disappeared and phagocytosis was important. My results are inconsistent with this study. I observed haemocyte clumps (incipient nodules) containing bacteria at each time point of the time course and at no stage did they disappear. HP cells were evident in the haemolymph as early as 30 min post-injection but were not observed by Horohov and Dunn (1983). This is likely to be due to the methodological and technological differences between the two studies.

HP cells were invariably associated with haemocyte microaggregates when haemolymph was examined from experimentally injected insects. Over 70% of all recorded microaggregates

were associated with HP cells. The majority of haemocytes within the microaggregates, which appeared to be GR, appeared to have phagocytosed none or very few bacteria. Phagocytosis within nodules by haemocytes is not a novel phenomenon (Horohov and Dunn, 1983) but my data suggest that phagocytosis by HP cells is intimately linked with nodule formation. Since bacteria-filled HP cells that are not associated with microaggregates were also observed, I suggest that phagocytosis by HP cells precedes HP cell association with microaggregates.

The results on microaggregate association with HP cells are consistent with the model put forward by Davies and Siva-Jothy (1991) for the initiation of encapsulation. According to this model HP cells would act as 'recognition' haemocytes, patrolling the haemocoel for microbial presence. During the bacterial infection, HP cells recognise and attach to large numbers of bacteria and then act as nuclei for subsequent nodule formation. Thus would require communication between HP cells and the other haemocyte types either at the cell surface level (a change in the adhesive properties) and/or at the intercellular signalling level (secretion of humoral factors). Eicosanoids (see chapter 6) are candidate signalling molecules known to be important in nodule formation. This constitutes a very effective and resourceful model because huge numbers of bacteria can be trapped by a single HP cell. This does not appear to be a universal mechanism however, as Ratcliffe and co-workers report that degranulation of GR within early nodules in *G. mellonella*, is responsible for the trapping of bacteria and cells fitting the description of HP cells were not reported (Ratcliffe and Gagen, 1976; Gagen and Ratcliffe, 1976).

This study provides descriptive and quantitative data on a novel hyperphagocytic haemocyte in *M. sexta*. I believe HP cells provide the immune system of this insect with the means to sequester large numbers of bacterial invaders and probably act as nuclei for nodule formation. The extreme levels of phagocytosis displayed by these cell types imply they are a key component in the microbial defensive strategy of *M. sexta* and therefore a likely target for immunosuppressive virulence factors. If HP cells are common in insect immunity, it would not be surprising to find entomopathogens that specifically target these cell types.

Chapter 4

Suppression of cellular immune responses in *M. sexta* by the entomopathogenic bacterium *Photorhabdus luminescens*.

*** Some of the data reported in this chapter is also presented and discussed in C.P. Silva *et al.* (2002) Cellular Microbiology, volume 4, pp. 329-339.**

4.1 Introduction

Photorhabdus luminescens is a Gram-negative bacterium that associates mutualistically with entomoparasitic nematodes of the family Heterorhabditidae. Upon invasion of the insect host, *P. luminescens* are released from the gut of the nematode into the insect haemocoel where they multiply quickly and death of the host usually occurs within 48 hours.

P. luminescens is a virulent insect pathogen that can kill the insect alone (i.e. without the nematode) following injection into the haemocoel (Silva *et al.*, 2002). As with all successful pathogens, this bacterium must employ mechanisms that enable it to avoid or depress the insect immune system. It is well established that the main defensive responses in insects to bacterial infection are phagocytosis and nodule formation (Ratcliffe and Walters, 1983). Because death of the insect usually occurs quickly after infection, it would appear that these immune responses are ineffective against *P. luminescens.*

The pathogenesis of *P. luminescens* is thought to involve a wide variety of virulence factors *in vivo* including toxin complexes (Tc; Bowen *et al.*, 1998), antibiotics (Hu and Webster, 2000), haemolysins (Brillard *et al.*, 2002), proteases (Schmidt *et al.*, 1988), lipases (Wang and Dowds, 1993) and lipopolysaccharide (LPS; Dunphy, 1995). It is unclear however, how these factors interact with the host's immune responses and their effects on insect haemocytes are not well documented. Most published work concerns the release of LPS into the haemolymph which has been shown to inhibit the activation of prophenoloxidase (Dunphy and Bourchier, 1992), an important enzyme in insect

immunity, and cause damage to insect haemocytes (Dunphy, 1995; Dunphy and Webster, 1988)

In this chapter, I describe an investigation of the interaction between *P. luminescens* and the cellular immune system of *Manduca sexta*. This study reveals that *P. luminescens* is able to produce factors *in vitro* that suppress both phagocytosis and nodule formation. It is also shown that *P. luminescens* is able to actively suppress its own phagocytic uptake by *M. sexta* haemocytes.

4.2 Results

4.2.1 Suppression of phagocytosis

4.2.1.1 Phagocytosis of *E. coli* and *P. luminescens*

Haemocyte monolayers were exposed to live and dead (heat-killed) *P. luminescens* and *E. coli* cells *in vitro* and after 2 hours the levels of phagocytosis by the haemocytes was assessed. This showed that live *P. luminescens* were able to inhibit their own uptake by the haemocytes (Fig. 1) whereas heat-killed *P. luminescens* were phagocytosed at a much higher level. The difference between phagocytosis of dead *E. coli* and dead *P. luminescens* was not significant ($P > 0.05$). Heat treatment of the *E. coli* had no significant effect on phagocytosis as both live and dead *E. coli* were phagocytosed at similar levels ($P > 0.05$, t-test). Viability of the haemocytes in these experiments was routinely assessed and remained above 90% in all cases.

4.2.1.2 Effect of *P. luminescens* W14 filtrate on phagocytosis

Cell-free supernatant of *P. luminescens* W14 (after growth in liquid broth for 48 h) exhibited an anti-phagocytic property toward insect haemocytes incubated with *E. coli*. This effect was dose-dependent (Fig. 2). The anti-phagocytic factor displayed significant activity in 1% diluted supernatant compared with the control broth in which no bacteria had grown ($P < 0.05$, t-test). There was no suppressive effect on phagocytosis by *E. coli* supernatant or liquid broth alone, compared to monolayers with no added broth.

The production of the anti-phagocytic factor in W14 supernatant occurred at the same time as bacterial growth in culture (Fig. 3). After 24-36 h in culture, the bacteria were growing exponentially and it was at this time that the supernatant first showed significant anti-phagocytic properties (at 10% dilution). The suppressive effect on phagocytosis increased further in 48 h supernatant, as the bacterial growth entered stationary phase.

4.2.1.3 Effect of boiling on W14 anti-phagocytic factor

Boiling W14 supernatant (10% dilution) for 3 min completely abolished its anti-phagocytic activity (Fig. 4). There was no significant difference between boiled filtrate and controls (liquid broth alone) for the phagocytosis of *E. coli* ($P > 0.2$). This suggests this factor is unlikely to be a small molecule. Boiled liquid broth alone had no suppressive effect on phagocytosis.

4.2.1.4 Anti-phagocytic properties of *P. luminescens* W14-infected plasma

Haemolymph from larvae infected with W14 (during late-stage infection) was sterile-filtered and the resulting plasma was assessed for anti-phagocytic activity (Fig. 5). Control plasma (from healthy insects) had no effect on the phagocytosis of *E. coli* by haemocyte monolayers. Infected plasma, on the other hand severely impaired the phagocytic activity of the haemocytes. It was found, however that most of the haemocytes exposed to the infected plasma exhibited unusual morphologies and the viability of the haemocytes also fell from over 90% (controls) to less than 20% (infected plasma). Thus, the observed effects of the infected plasma appeared to be due to the ill-health of the haemocytes. The results in Fig. 5 show levels of phagocytosis from the live haemocytes only, as dead haemocytes were not considered. Thus, the observed level phagocytosis by viable haemocytes was still very low in the presence of infected plasma.

4.2.2 Suppression of nodule formation

4.2.2.1 Influence of bacterial species on nodule formation

Intrahaemocoelic injections of both live and dead (heat-killed) *E. coli* provoked high levels of nodule formation at 24 h post-injection (Fig. 6). Dead *E. coli* provoked significant higher levels of nodule formation compared with live *E. coli* ($P < 0.05$). Control larvae (uninjected or injected with Grace's insect medium alone) possessed very few nodules and in most cases had none.

The melanised structures that were regarded as nodules *in vivo* were excised from larvae injected with FITC-labelled *E. coli* and viewed under fluorescence optics (Fig. 7). This procedure revealed that large numbers of *E. coli* were trapped by these structures and supported previous descriptions that they were indeed nodules (as defined by Miller *et al.*, 1994).

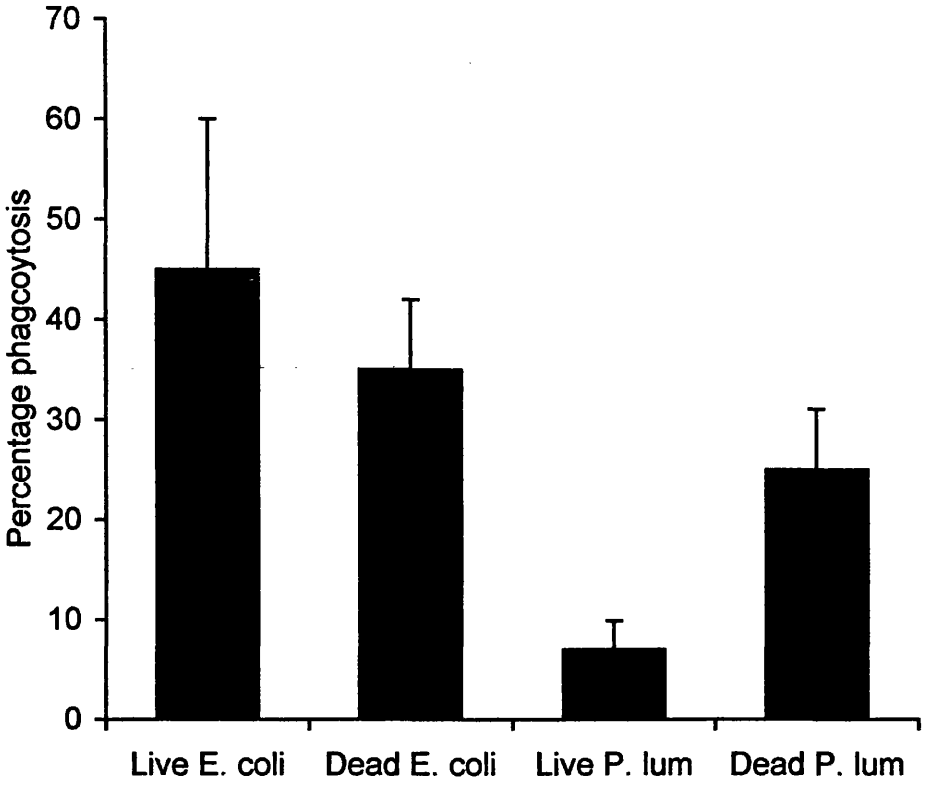
Heat-killed *P. luminescens* W14 provoked significantly lower levels of nodule formation than *E. coli* (Fig. 6). Furthermore, live W14 provoked very few nodules suggesting that this bacterium is not easily recognised by the insect or that it actively suppresses nodule formation.

4.2.2.2 Influence of *P. luminescens* W14 supernatant on nodule formation

Cell-free supernatant from 48 h *in vitro* culture of W14 had a strong suppressive effect on nodule formation when injected into larvae together with *E. coli* (Fig. 8). The effect of heating the supernatant (boiling for 3 min) had no affect on its anti-nodulation activity suggesting that the active principle was a different factor to that which suppressed phagocytosis.

Fig. 1. Percentage phagocytosis of *E. coli* or *P. luminescens* by *M. sexta* haemocyte monolayers. Both live and dead (heat-killed) *E. coli* provoked high levels of phagocytosis. Live *P. luminescens* was able to inhibit its own phagocytosis as heat treatment of this bacterium reduced the suppressive effect on phagocytosis. There was no significant difference between the phagocytosis of dead *E. coli* and *P. luminescens* ($P > 0.05$). Columns show means ± SD (over 6 microscopic fields of view per treatment) and the experiment was repeated twice.

Fig. 2. Dose-dependent effect of *P. luminescens* W14 broth culture (48 h) supernatant on phagocytosis of *E. coli* by *M. sexta* haemocytes. The anti-phagocytic factor became significantly active in 1% supernatant ($P < 0.05$ compared with controls). Six fields of view were assessed per treatment and the experiment was repeated twice. Points represent means ± SD.


70
60
50
40
30
20
10
0
Percentage phagcoytosis
Live E. coli
Dead E. coli
Live P. lum
Dead P. lum

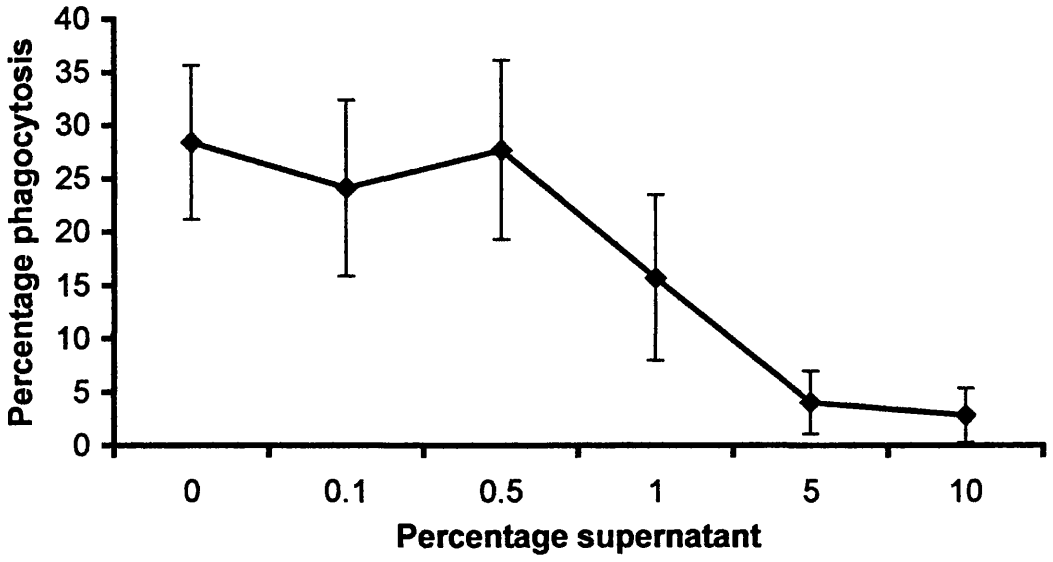
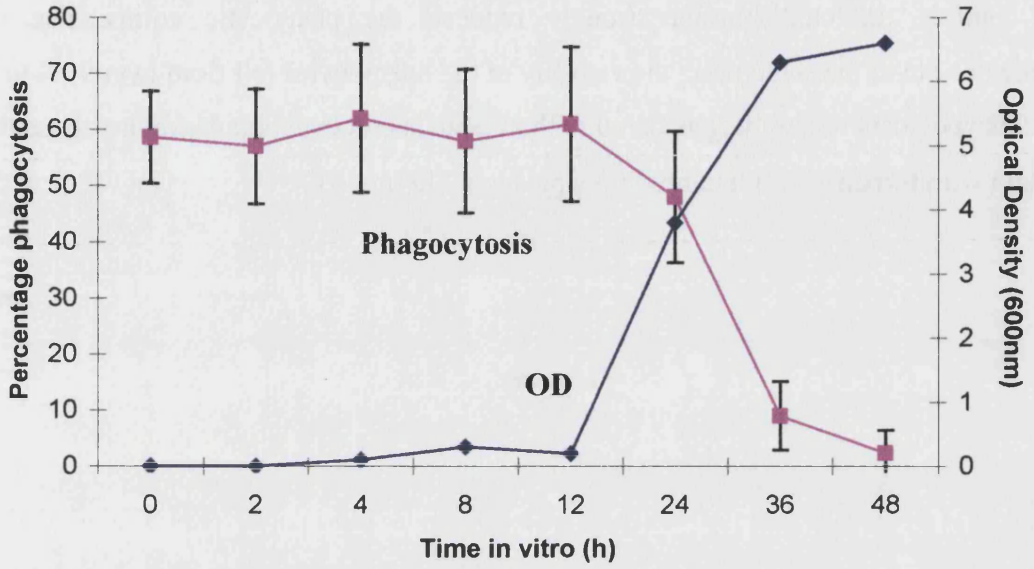

40
35
30
25
20
15
10
5
0
Percentage phagocytosis
0
0.1
0.5
1
5
10
Percentage supernatant

Fig. 3. *P. luminescens* W14 growth (OD) *in vitro* coincides with the production of an anti-phagocytic factor in the supernatant. Significant anti-phagocytic activity of the supernatant appeared during the exponential growth of the bacteria (between 24-36 h, $P = 0.000$) and this remained as the bacteria entered stationary phase (36-48 h). Points show means ± SD of the percentage phagocytosis by haemocyte monolayers exposed to the supernatant (6 fields of view were assessed per treatment and the experiment was repeated twice).

Fig. 4. Heating of W14 supernatant strongly reduced its anti-phagocytic properties. Supernatant and broth (at 10% dilution of original) were boiled for 3 min and then exposed to the haemocytes before the addition of *E. coli*. After 2 h, haemocytes in the presence of the boiled supernatant displayed similar phagocytic activities as haemocytes exposed to the broth alone. There was no significant difference between the broth treatments and boiled supernatant ($P > 0.05$). Columns show means ± SD. 10 fields of view were assessed per treatment.


Percentage phagocytosis
Optical Density (600nm)
Phagocytosis
OD
Time in vitro (h)
0
2
4
8
12
24
36
48

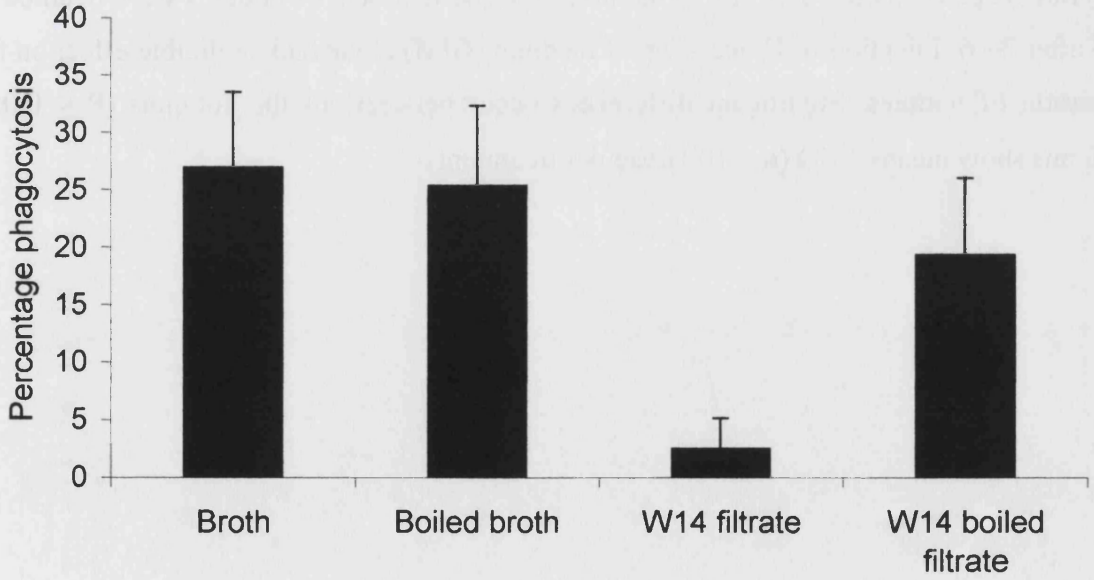
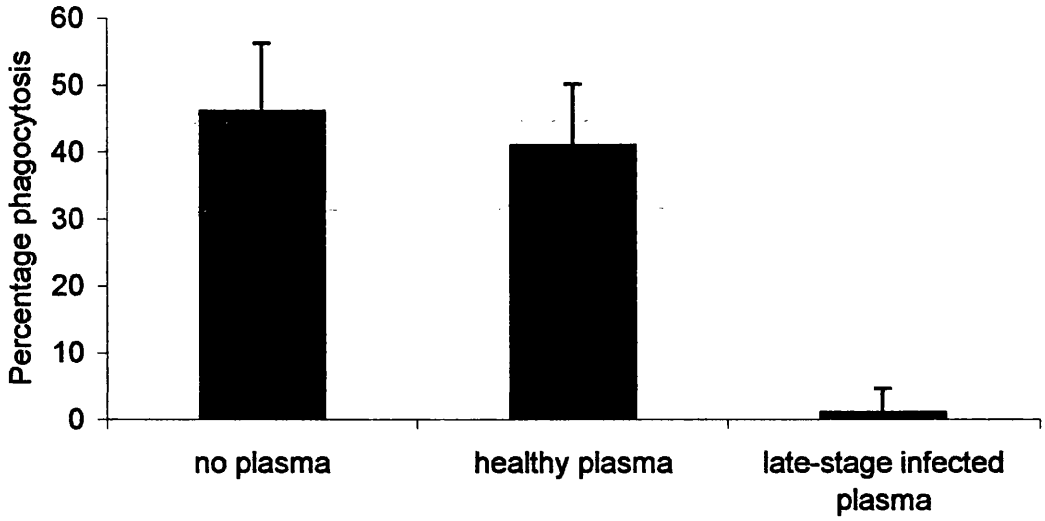
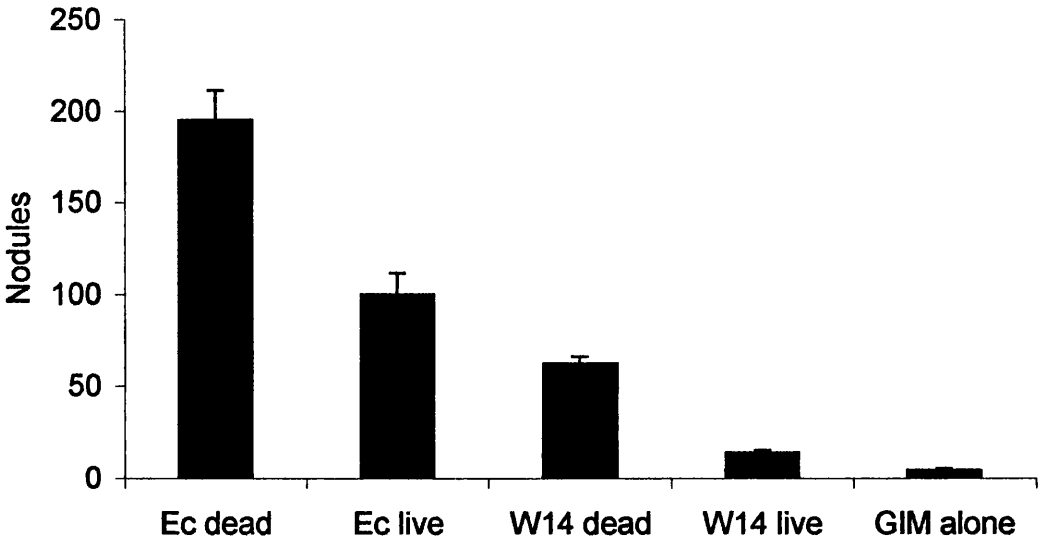
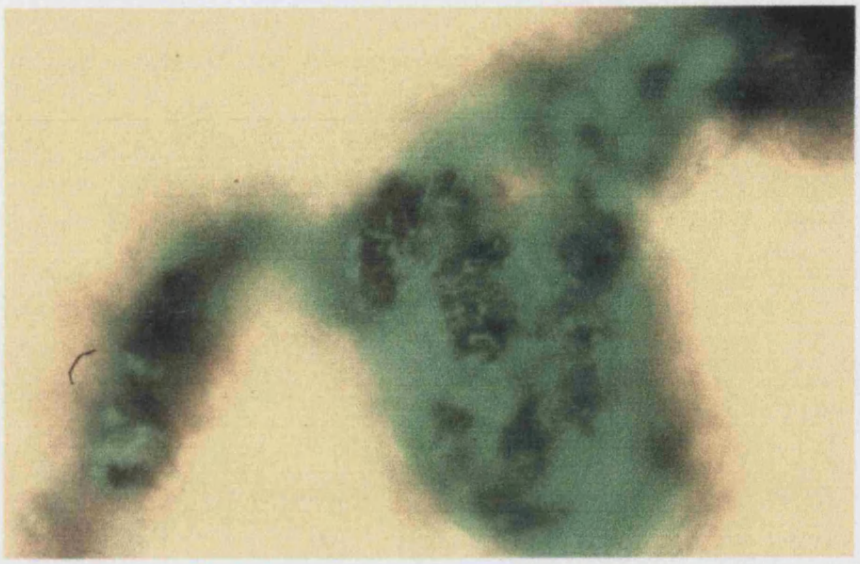

Percentage phagocytosis
Broth
Boiled broth
W14 filtrate
W14 boiled filtrate

Fig. 5. Effect of W14-infected plasma on the phagocytosis by haemocyte monolayers. Haemocytes were exposed to infected and non-infected plasma and then incubated with *E. coli*. No significant effect on phagocytosis was caused by non-infected plasma compared with controls. Infected plasma strongly reduced the phagocytic competence of the haemocytes but at the same time, the viability of the haemocytes fell from over 90% to below 20%. Phagocytosis was only considered in the viable haemocyte population i.e. those that did not stain with trypan blue. Columns show means ± SD (n = 6).

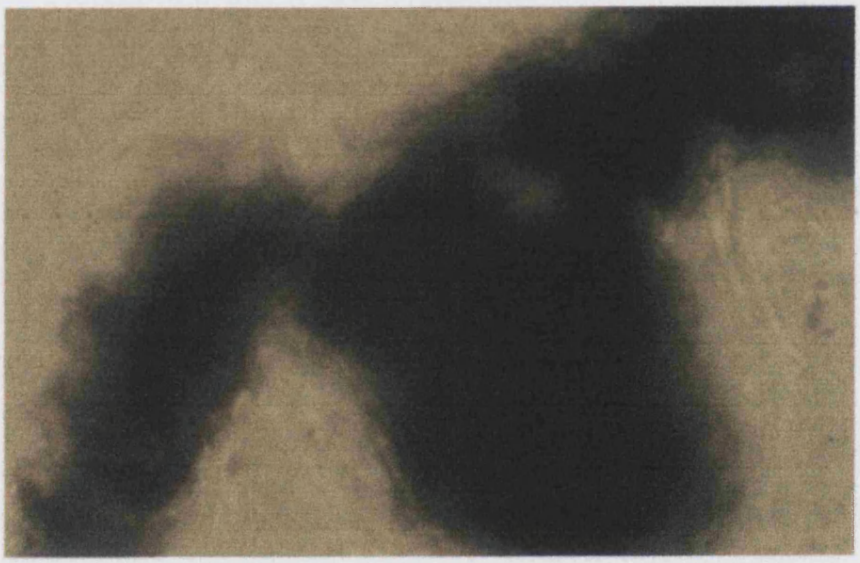
Fig. 6. Nodule formation *in vivo* at 24 h post-injection with 1 x 10^6 live and dead *E. coli* or *P. luminescens* W14 suspended in 100µl Grace's insect medium. Larvae were injected with the bacterial suspensions (as outlined in the text) and the numbers of nodules were counted *in vivo* after 24 h. Injection of Grace's insect medium (GIM) alone had negligible effect on the formation of nodules. Significant differences occur between all the columns ($P < 0.05$). Columns show means ± SD (n = 10 larvae per treatment).


Percentage phagocytosis
60
50
40
30
20
10
0
no plasma
healthy plasma
late-stage infected plasma


Nodules
250
200
150
100
50
0
Ec dead
Ec live
W14 dead
W14 live
GIM alone



Fluorescence (x 200)



Phase-contrast (x 200)

Fig. 7. A mature nodule excised from a dissected larva after injection with 1 x 10^6 FITC-labelled *E. coli*. UV illumination revealed that the melanised structures regarded as nodules *in vivo* had trapped an enormous number of bacteria (green).

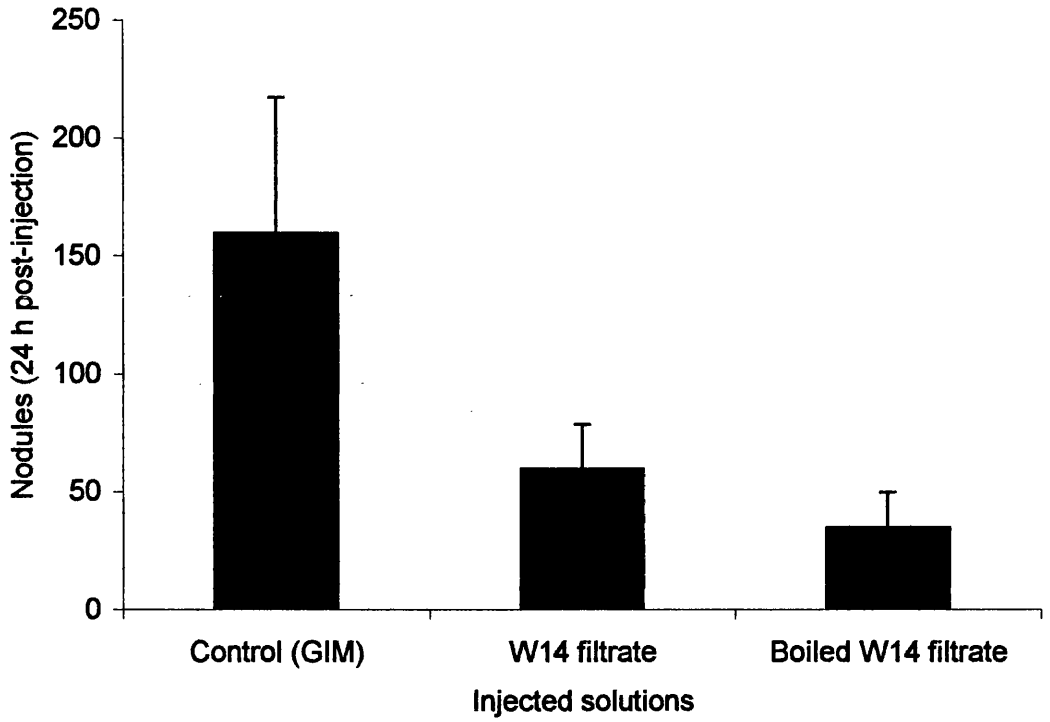


Fig. 8. Nodules formed *in vivo* in response to an injection of *E. coli* after 24 h post-injection. Grace's insect medium (GIM), W14 supernatant (48 h growth) and boiled W14 supernatant (48 h growth) were co-injected with the bacteria. Both heated and non-heated W14 supernatant had a highly significant suppressive effect on nodule formation ($P = 0.000$). Columns show means ± SE ($n = 10$).

4. 3 Discussion

Photorhabdus luminescens is a virulent insect pathogen. Upon infection, this bacterium is able to multiply quickly *in vivo* and kill the insect within 2-3 days. Thus, as with all true pathogens, *P. luminescens* must possess mechanisms that allow it to overcome the insect's immune responses. The two main barriers to bacterial infection in insects is phagocytosis and nodule formation. Therefore, I tested the ability of *P. luminescens* W14 to avoid or suppress these immune processes. Comparisons were made throughout this study between non-pathogenic *E. coli* and *P. luminescens* W14.

Live W14 were capable of avoiding both phagocytosis and nodulation compared to *E. coli.* When W14 were heat-killed they were phagocytosed avidly (at similar levels to *E. coli*) and provoked much higher levels of nodule formation than live W14 cells. Two possibilities suggest themselves. First, the bacteria may produce factor(s) that actively inhibit cellular immune responses by the host. Such factor(s) might be secreted into the culture medium and/or be retained on the surface of the bacterial cell. Second, the bacteria may produce factors that prevent their recognition as foreign objects. This seems less likely, as it is known that insect haemocytes can both phagocytose and encapsulate inanimate material such as polymer beads, but it is possible that the bacteria may be coated with factor(s) that mimics the molecular characteristics of the host. The fact that heat killed *P. luminescens* cells were recognized by the haemocytes and provoked both phagocytic and nodulation reactions, may be explained either in terms of the prevention of secretion of an immune-suppressing toxin(s) or in terms of the removal (or deactivation) of a disguising factor.

I tested the hypothesis that W14 produces secreted factors that inhibit phagocytosis and nodule formation. I found that W14 cell-free supernatant (when grown in liquid broth) was inhibitory to both immune responses. Similar anti-phagocytic effects of *P. luminescens* supernatant have been reported against locust haemocytes (Van Sambeek and Wiesner, 1999). As liquid broth alone or *E. coli* supernatant did not have a suppressive effect, I can conclude that factors, produced by W14 were specifically responsible for the immunosuppressive effects. The evidence also suggests that the anti-phagocytic and anti-nodulation factors are separate as both responded differently to

heating; the anti-nodulation factor was heat-stable, while the anti-phagocytic factor was not.

The anti-phagocytic property of the W14 supernatant was studied in some detail. The factor(s) appeared to be produced during the exponential phase of bacterial growth (24 h) and remained present in the medium as the bacteria entered stationary phase (48 h). I used 48 h filtrate to show that the anti-phagocytic factor is effective in a dose-dependent manner and caused a significant suppressive effect in 1% supernatant. Thus, the factor(s) are either very potent or are present in high concentration in the 48 h filtrate. The fact that boiling the supernatant destroyed the anti-phagocytic activity suggests that the factor responsible is unlikely to be a small molecular weight peptide. Small peptides produced by entomopathogenic fungi have been typically associated with immunosuppression (Boucias and Pendland, 1998).

P. luminescens is known to possess several genes with sequence similarities to haemolysins (ffrench-Constant *et al.*, 2000), thus it would not be surprising to find haemolytic activities on insect haemocytes. In all phagocytosis assays described in this study however, the viability of the haemocytes was constantly assessed by trypan blue exclusion (as described in the materials and methods section). The incubation of haemocytes with live and dead bacteria and exposure to W14 supernatant had no affect on the viability of the haemocytes with haemocyte viability remaining above 90% in all cases. Thus, the observed reduction in phagocytosis was not due to cytolysis of haemocytes.

Infected plasma (from late-stage W14 infection) killed most of the haemocytes in the monolayer. Although only viable haemocytes were considered in the percentage phagocytosis results, the overall ill health of the haemocytes may explain the loss in phagocytic ability. The cause of the effect on the haemocytes was not identified but possibilities include (a) endogenous metabolites released by damaged tissues or in response to the septicaemia (late-stage infected haemolymph may possess host metabolites that would be toxic to healthy conventional haemocytes) (b) factors produced by the pathogen; late-stage infected haemolymph is likely to contain a range of non-host metabolites that may be toxic to haemocytes. Indeed, Brillard *et al.* (2002) have shown

that a haemolysin occurs in the haemolymph prior to insect death and that this toxin is not required for pathogenesis but may have a role in symbiotic interactions with the nematode. Furthermore, Daborn *et al.* (2002) have reported that *P. luminescens* possesses a gene known that encodes for a toxin termed mcf (makes caterpillars floppy) which, when expressed in *E. coli,* can kill the insects upon injection of these bacteria. The mcf toxic protein contains sequence similarity to pro-apoptotic proteins and culture supernatants of *E. coli* that have been transformed with the mcf gene appear to cause apoptosis in insect haemocytes. Although it is unclear whether mcf is present in *P. luminescens*-infected insects, it is a potential candidate for the haemocyte death observed in the present study.

Unlike the phagocytosis response, there was a difference in the nodule formation to live and dead *E. coli,* with dead bacteria inducing more nodules. This is likely to be due to enhanced recognition of the dead bacteria rather than a suppression of the immune response by the live bacteria. Heat treatment of *E. coli* may expose cell surface patterns that would make the bacteria more immunogenic. Although this could also explain the finding why dead W14 cells provoke higher nodulation than live cells, the inhibitory effect of the W14 supernatant on nodule formation suggests secreted factors are involved.

P. luminescens is known to produce many factors *in vitro* and *in vivo* with putative roles in immunosuppression. Dunphy and Webster (1988) have reported that LPS produced by *Xenorhabdus* (= *Photorhabdus*) *luminescens,* is important in immune suppression of the host *Galleria mellonella.* LPS is present on the outer surface of the Gram-negative bacteria (Prescott *et al.*, 1996), and its presence there could explain why live W14 are not phagocytosed and do not provoke nodule formation. It may be that heat treatment either causes the loss of LPS from the bacterial cell surface or that the LPS is degraded which compromises its immunosuppressive effect. Sufficient LPS may also enter the culture medium and this may explain the anti-phagocytic effect of filtered culture supernatants. The heat labile nature of the anti-phagocytic property of *P. luminescens* culture filtrates would not be consistent with this as LPS from most Gram-negative bacteria is heat stable (Prescott *et al.*, 1996). However, the heat stability of the anti-nodulation factor in W14 culture filtrates suggest that it could be LPS. Clearly more experiments are required here.

It is possible that *P. luminescens*, like other pathogenic members of the Enterobacteriaceae, may operate a type III secretion system to manipulate host haemocytes. Type III secretion systems are essential for the pathogenicity of many bacterial pathogens (for review see Cheng and Schneewind, 2000) and allows the bacterium to inject virulence factors into host cells. Enteropathogenic *E. coli* (EPEC) inhibits phagocytosis of host cells by a type III dependent mechanism (Goosney *et al.*, 1999). Interestingly, genome sample sequencing of *P. luminescens* W14 suggests this bacterium may possess such type III secretion apparatus (ffrench-Constant *et al.*, 2000). Although the anti-phagocytic and anti-nodulation factor(s) appear not to require direct transport into host cells to exert their effect (as W14 supernatant alone suppressed both responses to *E. coli*), this does not rule out their delivery into host cells by live bacteria *in vivo*.

The experiments reported in this chapter were done before the discovery of the hyperphagocytic (HP) cell type reported in chapter 3. Therefore, the inhibitory effects of *P. luminescens* W14 on phagocytosis refer only to the phagocytosis generally undertaken by conventional haemocytes. At the time of writing it was uncertain whether *P. luminescens* was able to inhibit phagocytosis by HP cells. Preliminary evidence (C.P. Au, University of Bath, personal communication) indicates that *P. luminescens* W14 may remain subject to phagocytosis by HP cells. Although, it is uncertain whether the bacteria would survive phagocytosis by HP cells, there are plenty of precedents of other bacterial pathogens that survive engulfment by mammalian macrophages (Amer and Swanson, 2002). Thus, HP cells may represent a haven for *P. luminescens* during infection, allowing the bacteria to avoid existing cell mediated and humoral immune responses.

Interestingly, Silva *et al.* (2002) have shown that injected *P. luminescens* W14 proliferates within *M. sexta*, largely within a specialised niche associated with the midgut wall. The ability to avoid phagocytosis by plasmatocytes by occupying such compartments would possibly facilitate the survival of *P. luminescens* within the insect. However, if HP cells resemble vertebrate macrophages and are able to infiltrate into or are resident within host tissues, then phagocytosis of *P. luminescens* by HP cells would lead to a concentration of the bacteria at a number of sites such as the midgut wall, as described by Silva *et al* (2002).

This study provides evidence for immunosuppressive mechanisms of *P. luminescens* toward the insect host *M. sexta*. There are many studies that report similar suppressive effects by fungal pathogens (Boucias *et al.*, 1995; Gillespie et al., 2000; Griesch and Vilcinskas, 1998; Vilcinskas *et al.*, 1997b) and insect parasitoids (Beckage, 1998; Dupas *et al.*, 1996; Hayakawa, 1994; Richards and Parkinson, 2000). However, the relative ease and amenability of manipulating the *P. luminescens* genome provides us with a more effective tool in studying entomopathogen-host interactions. I have shown that this bacterium produces at least two factors *in vitro* that have immunosuppressive properties. The next step is to elucidate the molecular basis of these compounds and work is underway with *Photorhabdus* mutants to discover the genes responsible for the immunosuppressive factors.

Chapter 5

Microbial infection causes the appearance of very large blood cells with extreme spreading ability in monolayers of *Manduca sexta*

5.1 Introduction

The ability to spread is a typical property of both vertebrate and invertebrate blood cells. In insects, it is generally accepted that upon recognition of nonself, the response of the haemocytes is to spread (Gillespie *et al.*, 1997). If the foreign agent is small, this spreading response results in the phagocytosis of the particle (see Chapter 6), whereas a larger foreign object (or many small foreign objects) would be encapsulated (or nodulated). Suppression of haemocyte spreading is a common strategy employed by many insect pathogens and parasites and implies a particular of importance of haemocyte spreading in insect defence (Davies *et al.*, 1987; Griesch and Vilcinskas, 1998; Hung *et al.*, 1993; Strand and Noda, 1991). Many endogenous signals, thought to be released by haemocytes or wounded tissue (Clark *et al.*, 1997), also influence haemocyte spreading.

The presence of nonself in the insect haemocoel may result in a range of responses including changes in the haemocyte population (Horohov and Dunn, 1982), changes in the state and behaviour of the haemocytes (i.e. spreading, phagocytosis and nodule/capsule formation), induction of antimicrobial peptides and proteins (Hoffmann *et al.*, 1993) and activation of the prophenoloxidase cascade (Marmaras *et al.*, 1996). In terms of haemocyte behaviour, spreading is a commonly assessed parameter during a microbial infection as it provides an indication of fitness of the immune system.

The present work shows that fungal or bacterial infections in *M. sexta* larvae cause the appearance of haemocytes with extreme spreading ability. Monolayers from uninfected larvae do not possess these cell types. The appearance of these hyperspreading cells is not a pathological effect of infection but a direct response of the immune system. The evidence is also consistent with a role for these hyperspreading haemocytes in nodule formation.

5.2 Results

5.2.1 Monolayers from healthy and infected larvae

Washed haemocytes were used to prepare monolayers from healthy larvae and larvae infected topically with the fungal pathogen, *B. bassiana* isolate 304. Larvae were bled on successive days following infection and the monolayers were stained with FITC-labelled phalloidin to visualise the cytoskeleton. All haemocytes were then examined by confocal microscopy.

Most adherent haemocytes in non-infected monolayers (Fig. 1A) were identified as plasmatocytes (PL) or granular cells (GR). PL were pleiomorphic spreading cells and commonly possessed several extending filopodia and a well spread nucleus. Confocal optical sections showed PL to be relatively flat. Granular cells on the other hand were rounded with a small diameter and possessed a nucleus that appeared to take up most of the cytoplasm. Although other haemocytes types exist in *M. sexta* (such as oenocytoids, spherulocytes and prohaemocytes), these were seldom observed in the monolayers but were considered in the total haemocyte counts.

Hyperspread haemocytes appeared in monolayers prepared from mycosed insects on day-3 post-inoculation with *B. bassiana* 304 (Fig. 1B). These cell types were rarely observed in monolayers from healthy larvae. The novel haemocytes or Very Large Blood Cells (VLBC), were much bigger than conventional PL and GR (identified using the criteria above), and usually took up the majority of space on the monolayer. As a consequence they were commonly found in close association with each other and other haemocytes. The circumference of the different cell types was measured using confocal software and revealed that VLBC were significantly larger than the other cell types ($P < 0.01$, t-test, Fig. 2).

There were two types of VLBC. Both lacked filopodia (thus the cell periphery was smooth and rounded) and had well spread nuclei. The smaller of the two types had a 'fried egg' shape (Fig. 1B), with most of the cytoplasm in the centre of the cell and a much thinner cytoplasm at the periphery. Nucleic acid (RNA and DNA), mitochondrial and tubulin

staining all showed these cellular components concentrated in the centre of the cell while the periphery comprised a thin layer of actin. The other VLBC type was speckled when stained with phalloidin and usually spread to a huge diameter (Fig. 3). These 'speckled' cells were extremely flat and usually fluoresced weakly due to low levels of actin across the cell. Without the use of phalloidin staining and confocal microscopy, it was difficult, and in many cases impossible to visualise both VLBC types due to their extreme thinness (Fig. 4). Efforts to observe them with conventional phase-contrast microscopy were largely unsuccessful. Both VLBC types, in particular the speckled cells, were only recognized using phase-contrast microscopy by a small lump in the nuclear region of the cell (presumably the nucleolus). Cryo-SEM (scanning electron microscopy) confirmed that the cells were very thin (Fig. 5) and provided an estimate of the measure of thickness, between 0.2 -1.0 μm at the periphery.

The two VLBC morphologies appeared in different ratios in monolayers. The VLBC with a fried egg morphology was clearly more abundant and represented 73.2% of VLBC whereas the speckled cell types represented 26.7% (200 VLBC cells counted over 30 different fields of view).

5.2.2 Time course of *B. bassiana* 304 infection

Very large blood cells were initially found in monolayers from larvae on day-3 following infection with *B. bassiana* 304. Subsequently, monolayers were examined on successive days post-inoculation and, at the same time, the haemolymph from infected larvae was plated out on agar medium to determine the number of fungal blastospores present. The results are given in Fig. 6 and show VLBC appeared early in the monolayers, just prior to fungal proliferation. VLBC numbers were at a maximum on day-2 post-infection and declined to zero by day-4 post-infection. This decline was concomitant with the increase in the growth of the fungus *in vivo*. VLBC were never found in monolayers heavily infected larvae although other haemocyte types such as conventional PL and GR were present (as shown in Fig. 1B).

5.2.3 Spreading of very large blood cells *in vitro*

Initially it was unclear whether VLBC were also large *in vivo.* To elucidate this, monolayers from infected larvae (at day 3 post-infection) were fixed after selected periods of time (2, 6, 10, 60 and 120 min) and the circumferences of spread haemocytes were measured using confocal software (the complete time course is given in Fig. 7).

At 2 min *in vitro*, all the haemocytes were of similar size and were small in diameter (Fig. 8). It was clear that by 6 min, VLBC spreading was rapid and markedly different to the other haemocyte types. During the spreading phase (10-60 min), many cells possessed filopodia but these were lost by 60 and 120 min when VLBC appeared to have reached maximum size and exhibited a rounded cell periphery (Fig. 8). These results show that VLBC appeared large *in vitro* because of their ability to spread extensively on the glass surface and that their native morphology *in vivo* was small and rounded, similar to other haemocyte types.

5.2.4 Effect of laminarin on haemocyte profile *in vivo*

Following the injection of laminarin, the larval cuticle melanised rapidly (within 20-60 min, Fig. 9A) but the haemolymph remained unchanged (in terms of colouration) until late into the time course (i.e. 48 h post-injection, Fig. 9B). The results of the time course are given graphically in Fig. 10A. The haemocyte profile between laminarin-injected and control larvae was very similar prior to 17 h post-injection. By 17 h post-injection, however, there was a marked change in the monolayer as almost all GR appeared to have degranulated (Fig. 11A). Degranulated GR were clearly visible by phase-contrast microscopy and appeared very different to regular GR upon phalloidin staining, in having a larger diameter and a decreased concentration of actin (Fig. 11C).

After a delay of 24 h, the very large blood cells (VLBC) typically associated with fungal infection were present in large numbers on the monolayer (Fig. 10A). The degranulated GR still remained but were interspersed with VLBC and PL. Unlike the complete loss in VLBC associated with the fungal infection, the VLBC in laminarin-injected larvae declined less rapidly. In all GIM-injected controls, no VLBC were observed.

Degranulation of GR occurred in all laminarin-injected larvae. By 36 h post-injection, few GR could be seen and PL (and VLBC) were the main cells present in the monolayer when viewed with confocal microscopy. At 12 h post-injection there was also a large change in plasma protein profile of the haemolymph as assessed by SDS-PAGE. There was a clear increase in protein concentration in the region of 62 kDa (Fig. 10C). This change thus preceded the degranulation of GR and induction of VLBC.

5.2.5 *In vitro* induction of VLBC

All attempts to induce hyperspreading *in vitro* from haemocytes in healthy monolayers were unsuccessful. Monolayers were made as before and were then exposed to various elicitors including fungal filtrate, infected plasma (at different stages of infection), *B. bassiana* 304 conidia and laminarin. All were tested at a range of concentrations and for different periods of time. An increase in haemocyte spreading could not be induced in control monolayers by any of the treatments. However, there was an observed inhibitory effect on haemocyte spreading caused by late-stage infected plasma and fungal filtrate, both of which often caused many of the haemocytes to round up or even detach from the coverslip.

Unlike that seen in the *in vivo* laminarin-injection experiment, degranulation of GR was not observed *in vitro* in haemocytes exposed to a range of concentrations of laminarin. Monolayers, examined after exposure to the laminarin solutions for 2 and 24 h displayed no degranulation at these different times (Fig. 11B).

No induction of VLBC occured in haemocyte suspensions treated with potential elicitors before their attachment to the coverslip. Subsequent monolayers from these treated cell suspensions did not appear markedly different to controls. In addition, coating the coverslips with plasma (at different concentrations) from larvae at different stages of infection had no effect on the spreading ability of subsequent monolayers.

5.2.6 Infection of larvae with other pathogens and parasites

VLBC were present in larvae infected with other microbial pathogens and non-pathogens including another isolate of *B. bassiana* (2253), a different fungal species (*Metarhizium anisopliae* isolate ME1), an entomopathogenic bacterium *(Photorhabdus luminescens* strain W14) and non-pathogenic heat-killed *Escherichia coli* (strain SOLR). No quantitative data was obtained from these results but each microorganism was marked as positive or negative for VLBC.

Larger parasites (the entomopathogenic nematode *Steinernema feltiae* and the parasitoid wasp *Cotesia congregata*) did not cause the induction of VLBC in the present study. A complete time course using these parasites was not performed, thus the results require confirmation.

5.2.7 VLBC and microaggregates (incipient nodules)

VLBC from infected or laminarin-injected larvae, were often associated with haemocyte microaggregates. In monolayers from insects 3 days post-inoculation with *B. bassiana* 304, all microaggregates (minimum of 5 counted for each of 4 infected larvae) were associated with hyperspreading cells (Fig. 12). However, most VLBC were solitary.

SEM revealed that VLBC (particularly the 'fried egg' type) form overlapping confluent cell layers in places on the coverslip, as each cell was tightly apposed to the next (Fig. 13). This behaviour of VLBC is characteristic of the layering and flattening of haemocytes that is thought to occur during nodule formation.

5.2.8 Labelling of very large blood cells with monoclonal antibody MS13

The monoclonal antibody MS13 specifically labels conventional plasmatocytes and not granular cells (Willot *et al.*, 1994). We confirmed this in a separate study (Fig 14A, Chapter 6) and also revealed that VLBC were labelled with this antibody (Fig. 14). This suggests that conventional plasmatocytes and VLBC share the 90 kDa cell surface protein that MS13 is known to specifically label (Wiegand *et al.*, 2000).

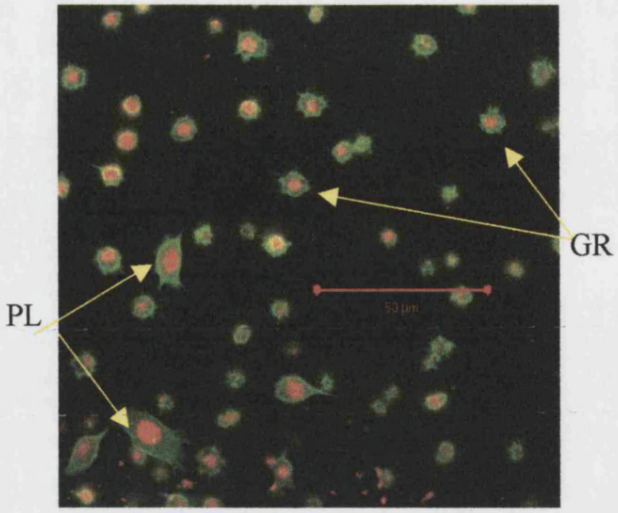


Fig. 1A. Control monolayers (from untreated larvae) stained with FITC-labelled phalloidin to visualise the F-actin cytoskeleton (green) and propidium iodide to visualise the nucleus (red). Most cells on the monolayers are granular cells (GR, smaller, rounded) and plasmatocytes (PL, pleiomorphic, spreading cells with filopodia). Haemocyte spreading did not differ markedly between monolayers from different insects. Bar = 50 μm.

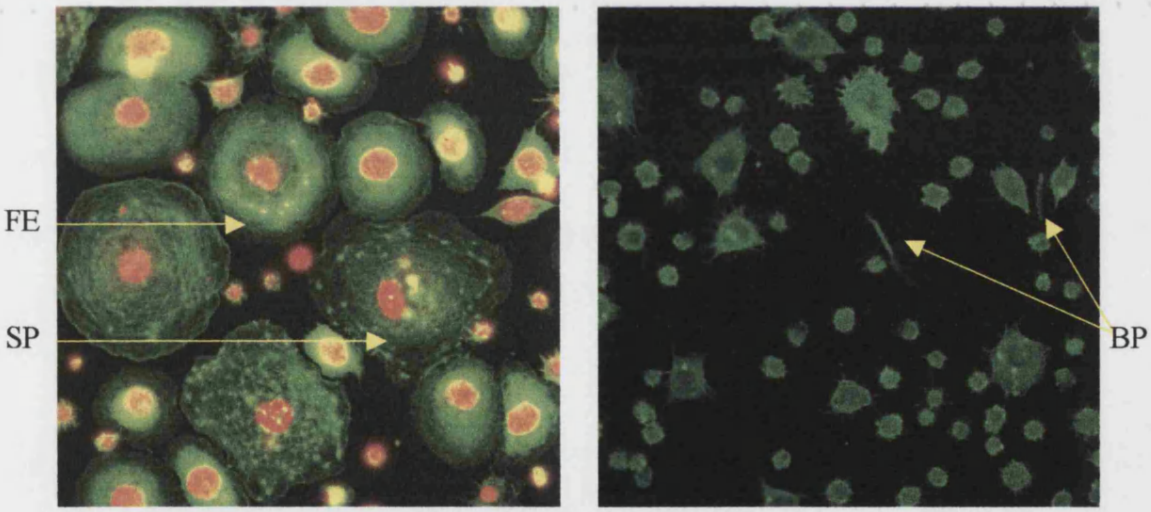


Fig. 1B. Monolayers from larvae infected with *B. bassiana* 304 on day-3 (left) and day-5 (right) post-infection. Haemocytes on day-3 post-infection show a much higher degree of spreading than controls. Two types of very large blood cells were evident: the smaller 'fried egg' (FE) type and the larger, more spread 'speckled' (SP) cell type. By day-5 post-infection, the spreading cells were never present. BP = fungal blastospores. Red = nucleus. Green = F-actin cytoskeleton. All figures are to scale.

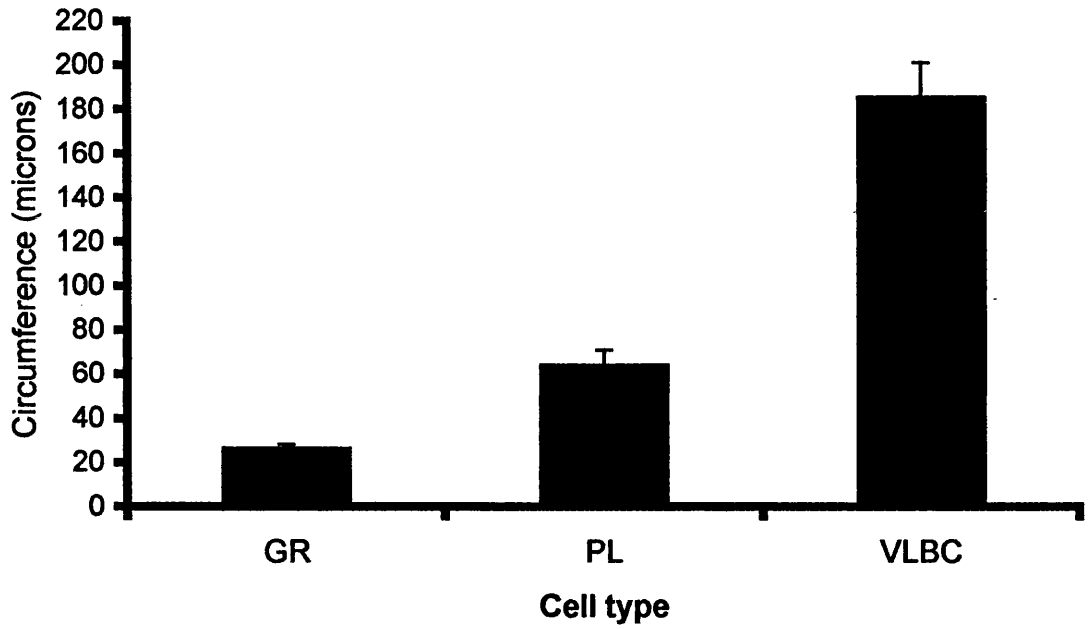


Fig. 2. Circumferences of haemocytes after 1 h incubation on glass coverslips *in vitro*. Monolayers were made from the haemocytes of larvae at day 3 post-inoculation with *B. bassiana* 304. Circumferences were determined by confocal software. There was a significant difference between the sizes of all 3 haemocyte types ($P < 0.01$, t-test, $n = 40$ cell types). Columns show means ± SD.

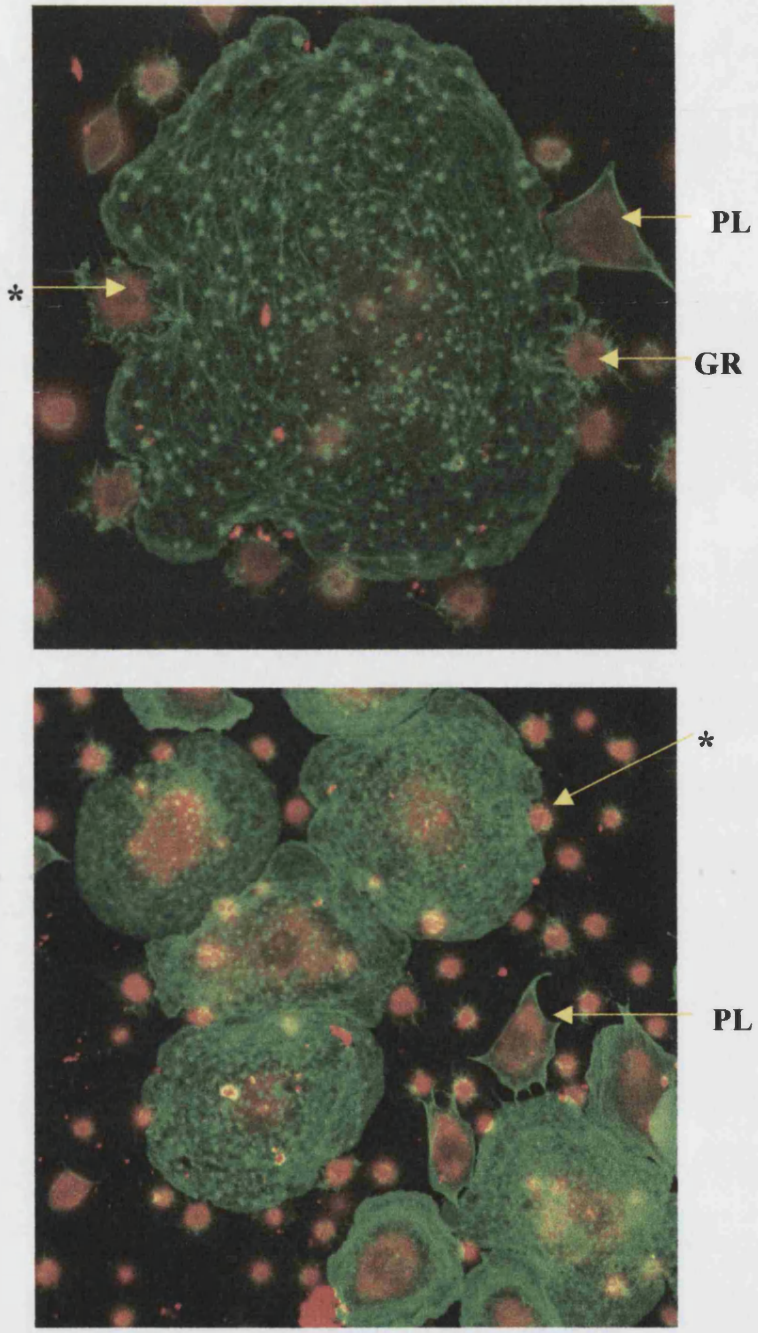


Fig. 3. The 'speckled' cell types of VLBC were found extensively spread and thus appeared very large. Other conventional haemocytes (i.e. PL and GR) can be seen close to the VLBC and in some cases impede its spreading as shown by (*). Red = total nucleic acid staining, Green = F-actin cytoskeleton.

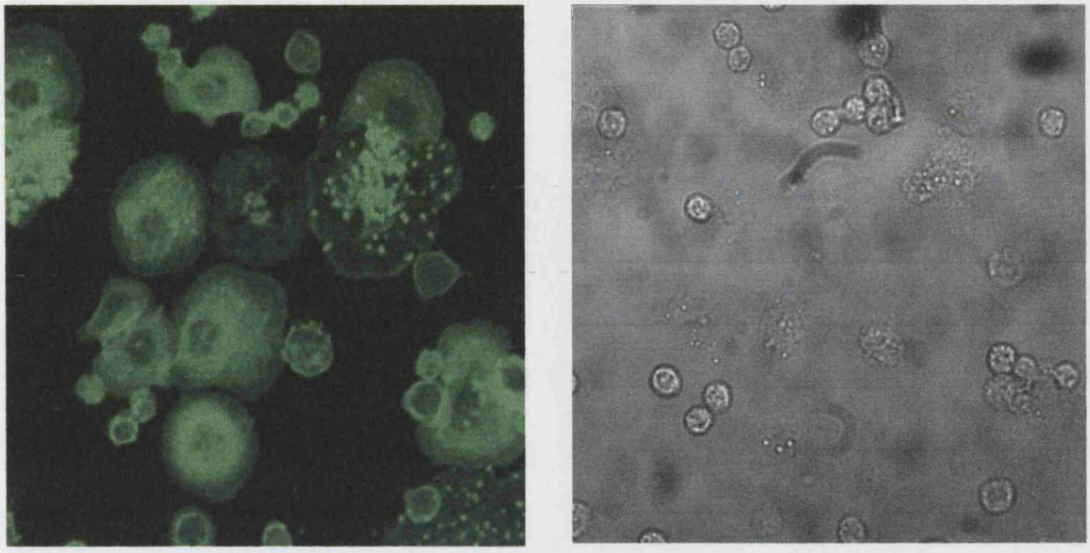


Fig. 4. The spreading cells associated with infection were difficult to visualise with conventional phase contrast microscopy (right) due to extreme thinness. Phalloidin staining (left) revealed that these cells often covered much of the coverslip. This may be one reason why these cell types have not been visualised previously.

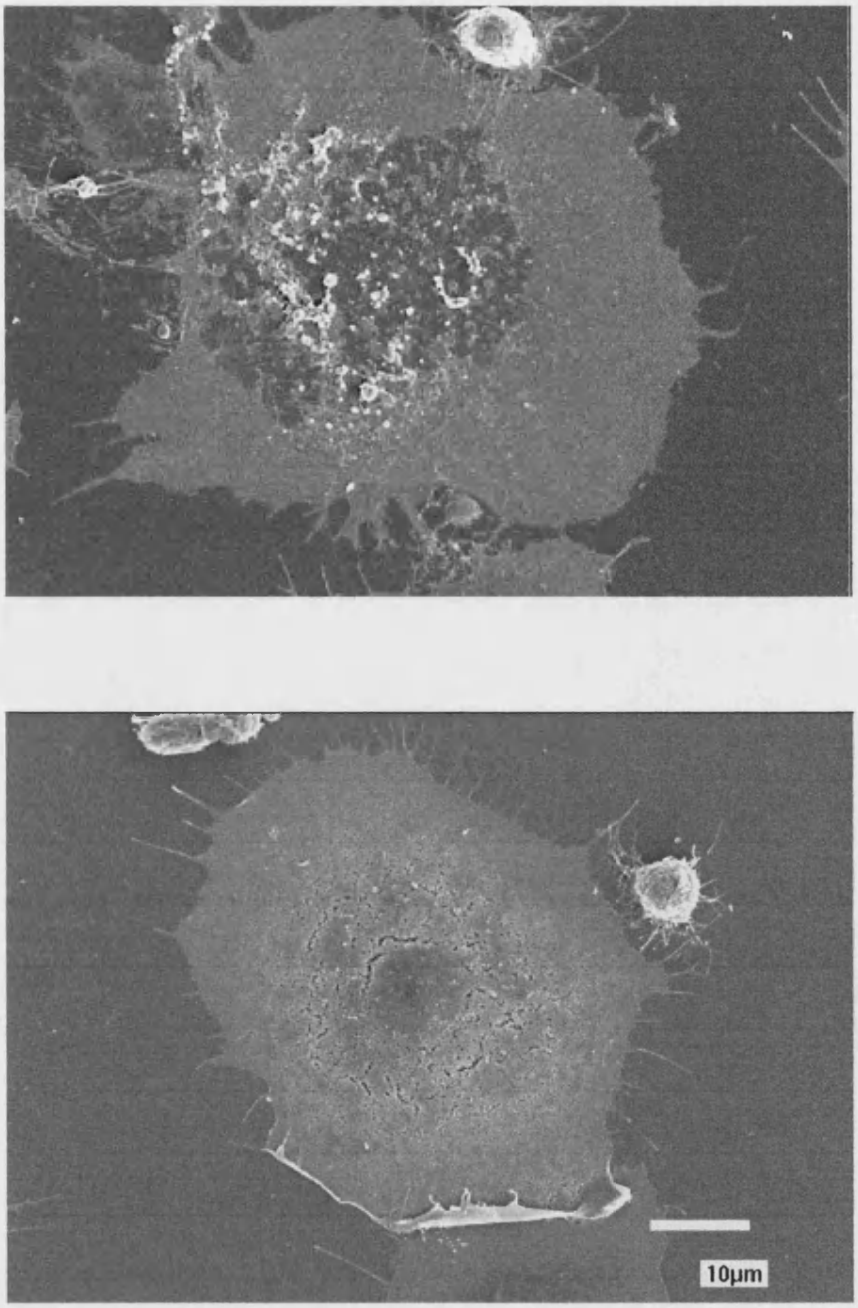


Fig. 5. Scanning electron micrograph of the two types of very large blood cell that appear during an infection with *B. bassiana* 304. Monolayers were prepared on day-3 post-infection. The larger 'speckled' cell type (top) and the 'fried egg' cell type (bottom) are both shown. Both are very thin and this is revealed in the bottom figure as the cell has partially curled over during SEM preparation. Both figures are to scale.

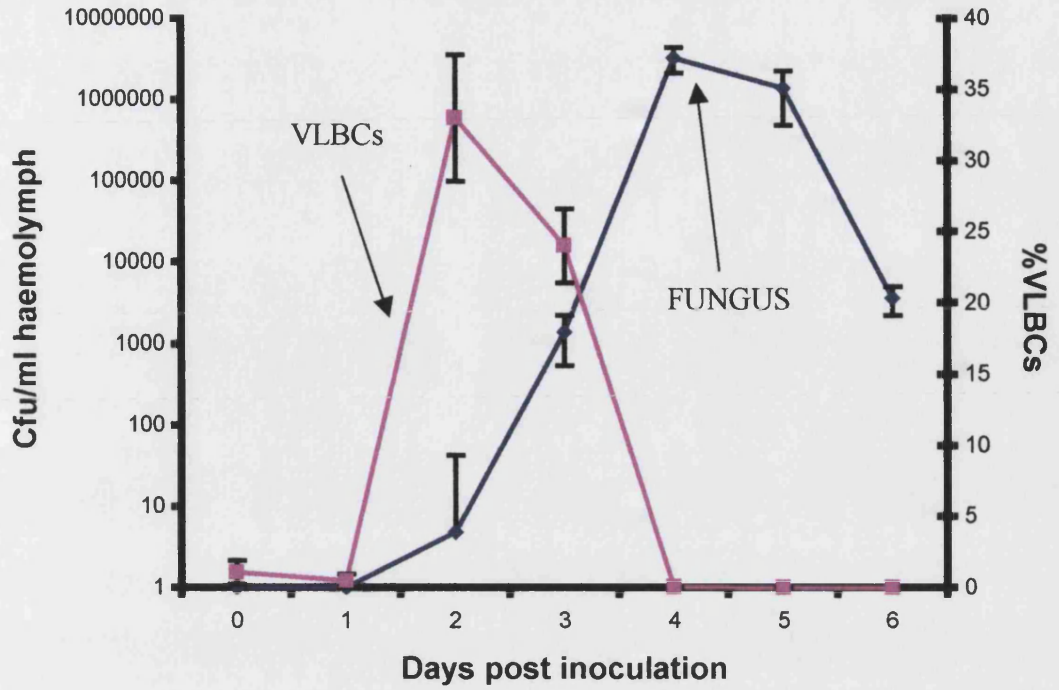


Fig. 6. VLBC numbers (expressed as percentage of the adherent haemocyte population) in monolayers from larvae infected with the fungal pathogen *B. bassiana* 304. Healthy larvae did not possess many VLBCs (and most larvae possessed none). By day 2 post-infection there was a considerable increase in the numbers of these cells in the monolayer. The levels of VLBC fell to zero by day-4 and this is concomitant with the growth of the fungus in the blood. Fungal growth was measured by plating blood, diluted in saline, out on Sabouraud's dextrose agar. It was assumed that colonies arose from individual blastospores and results are expressed as numbers of colony forming units (cfu). Points represent mean ± SE (n = 6 larvae).

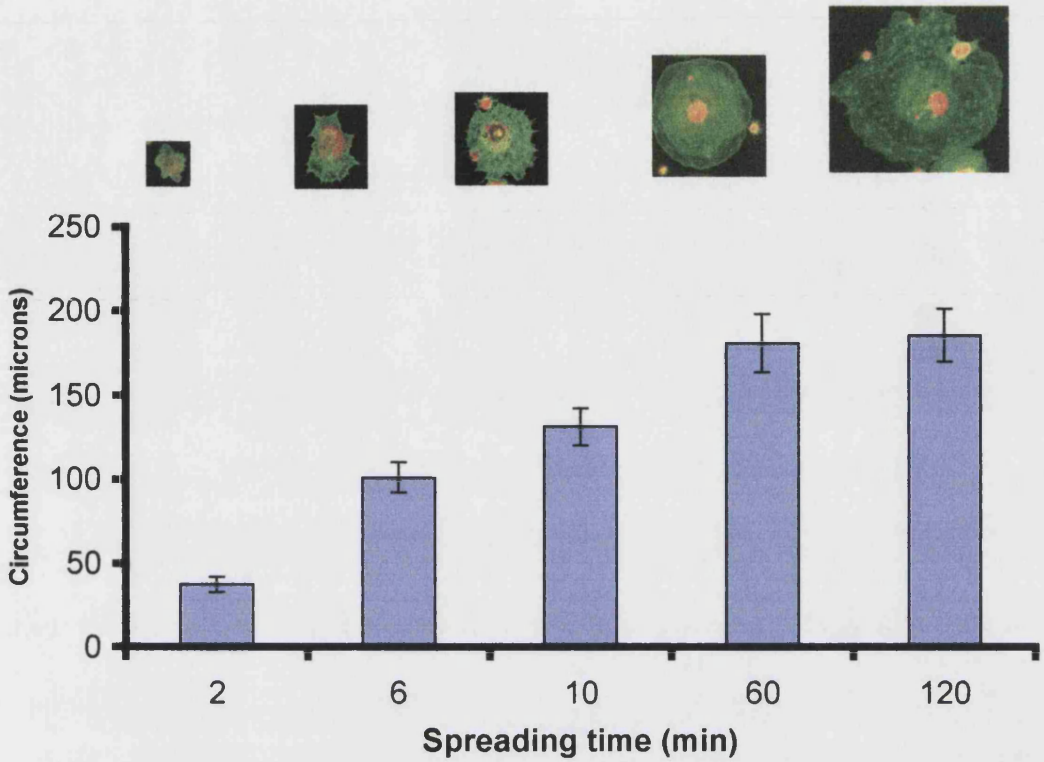
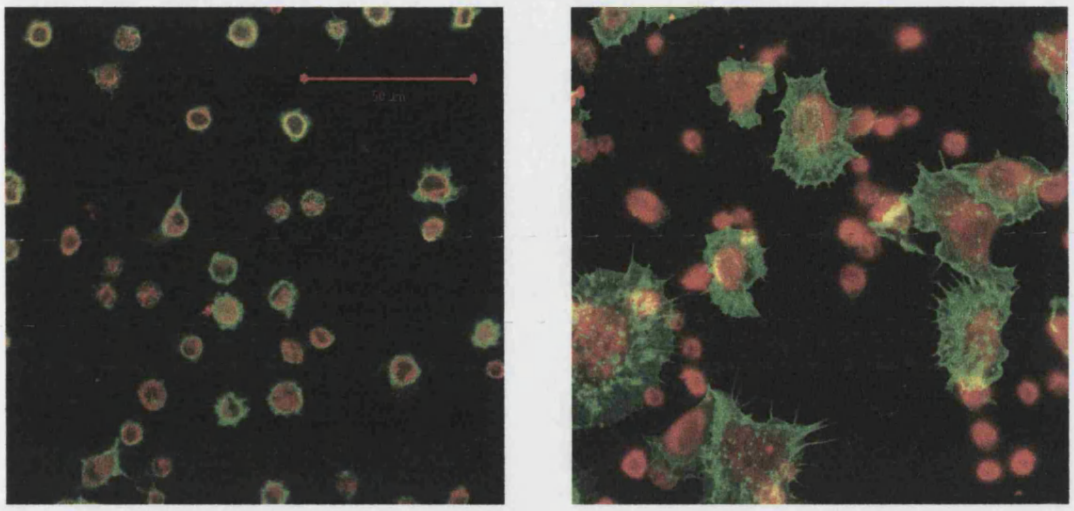
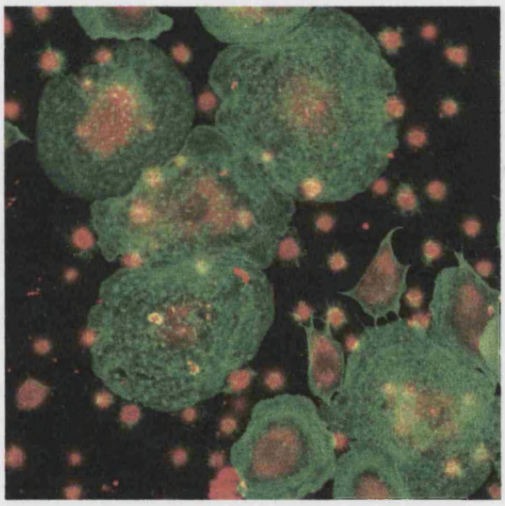


Fig. 7. VLBC spread rapidly and to a large size *in vitro*. Monolayers were prepared from fungal-infected larvae on day-3 post-infection. The monolayers were fixed at the shown time and then stained with FITC-labelled phalloidin. At 2 min *in vitro* all haemocytes appeared similar n size. By 6 min there was a clear difference between VLBCs and the other haemocyte types (see also Fig. 8). Spreading increased significantly at each time point ($P < 0.05$, t-test) up to 60 min *in vitro*. There was no significant difference between the sizes of VLBCs at 60 and 120 min ($P = 0.41$, t-test). Columns show means ± SE ($n = 15$ cells per time point).



2 min 6 min



60 min

Fig. 8. Spreading of haemocytes at different times *in vitro* in monolayers from fungal-infected larvae on day-3 post-infection. Haemocytes were fixed at the selected times and were then stained with FITC-labelled phalloidin and propidium iodide (without RNAase for nucleic acid staining). Results of spreading time course are given in Fig. 7. Green = F-actin cytoskeleton, Red = mitochondrial staining. All figures are to the same scale. Bar = 50 μm.

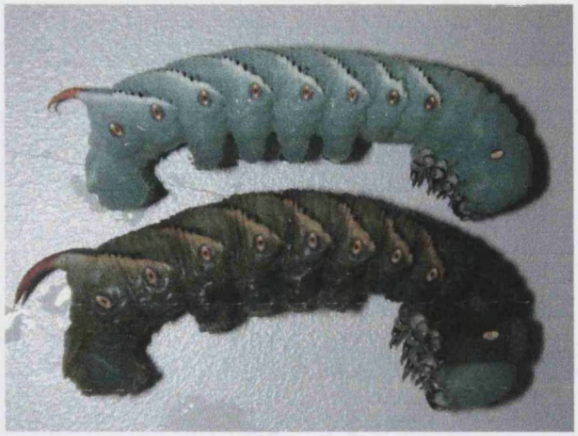


Fig. 9A. Laminarin-injected larvae (bottom) melanised quickly (20-60 min) following a 25 µl injection of 0.5% laminarin. Control larvae (top) injected with Grace's insect medium did not melanise.

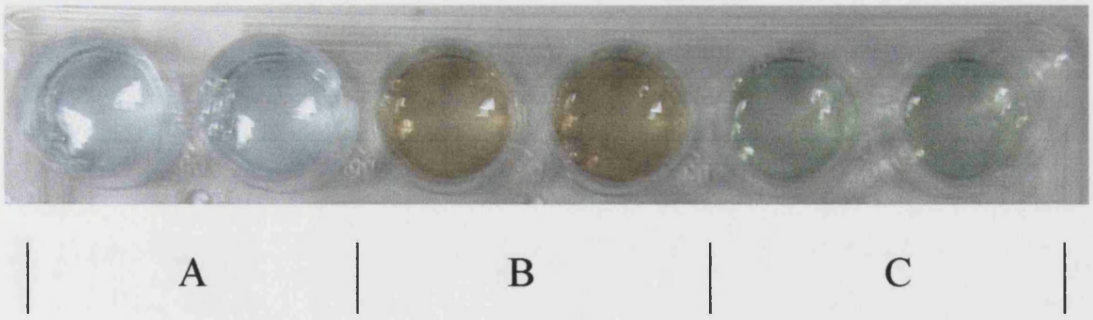


Fig. 9B. Colour of haemolymph from healthy larvae (A), larvae infected with *B. bassiana* isolate 304 at day-5 post-infection (B) and larvae injected with 25 µl 0.5% laminarin solution at 72 h post-injection (C). There was little change in the colour of the haemolymph between healthy larvae and laminarin-injected larvae up until 48 h post-injection. At this point the haemolymph from the laminarin-treated larvae started to appear slightly greenish in colour.

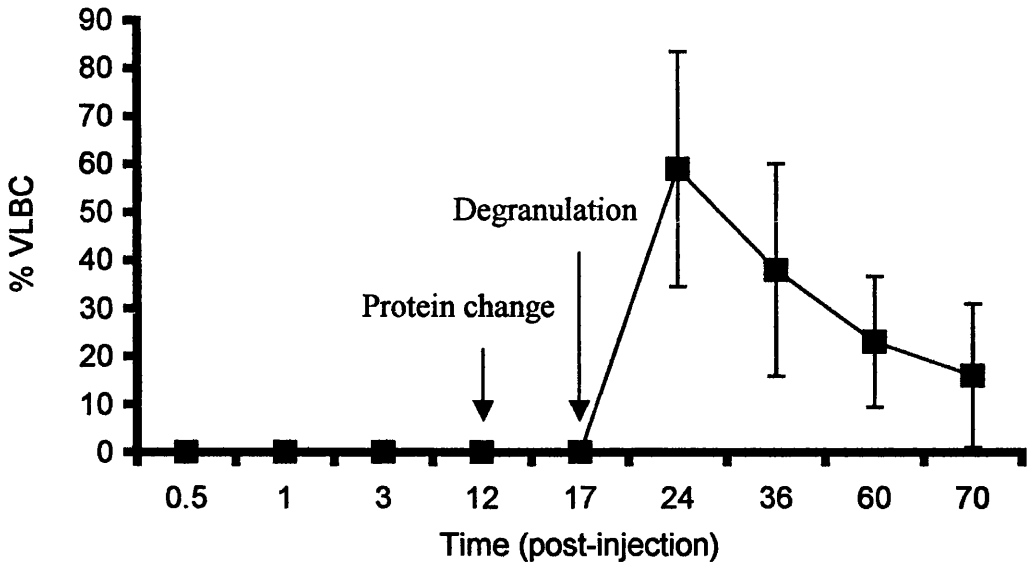


Fig. 10A. Percentage of VLBC in monolayers from larvae injected with 25 µl of 0.5% laminarin solution at different times post-injection. Arrows indicates the points of change in the protein profiles of the haemolymph and degranulation of the granular cells. Following these events, VLBC appear in the monolayers at 24 h and their numbers subsequently decrease progressively up to 70 h post-injection. Six larvae were assessed for each time point and the experiment was repeated on two occasions. A minimum of 500 haemocytes were counted per larva. Points show means ± SD.

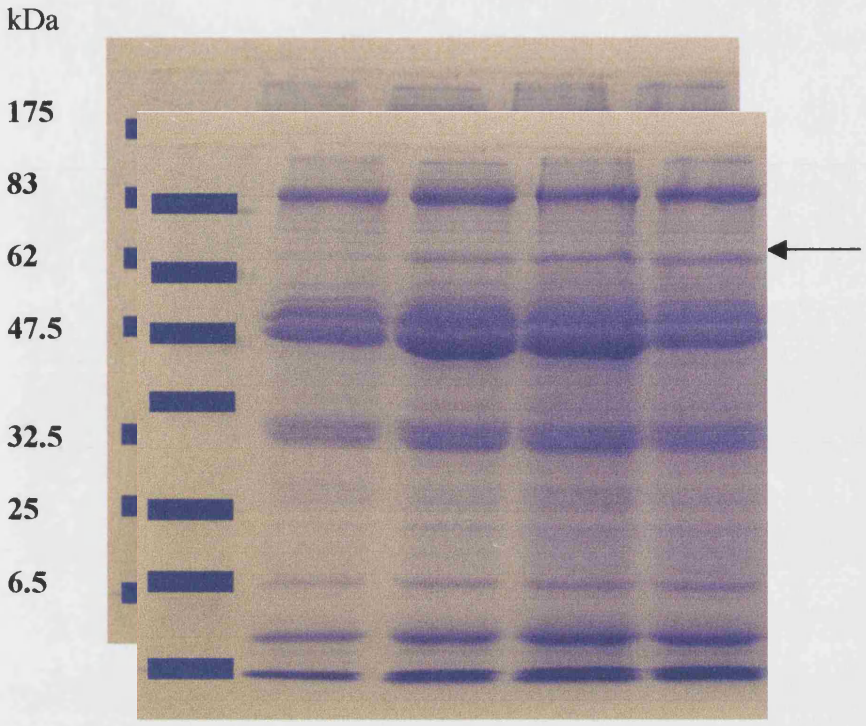


Fig. 10B. SDS-PAGE of haemolymph samples from larvae injected with a solution of laminarin at different times post-injection (indicated at the bottom of gel). At 12 h and 17 h a protein of approx. 62 kDa increased in concentration.

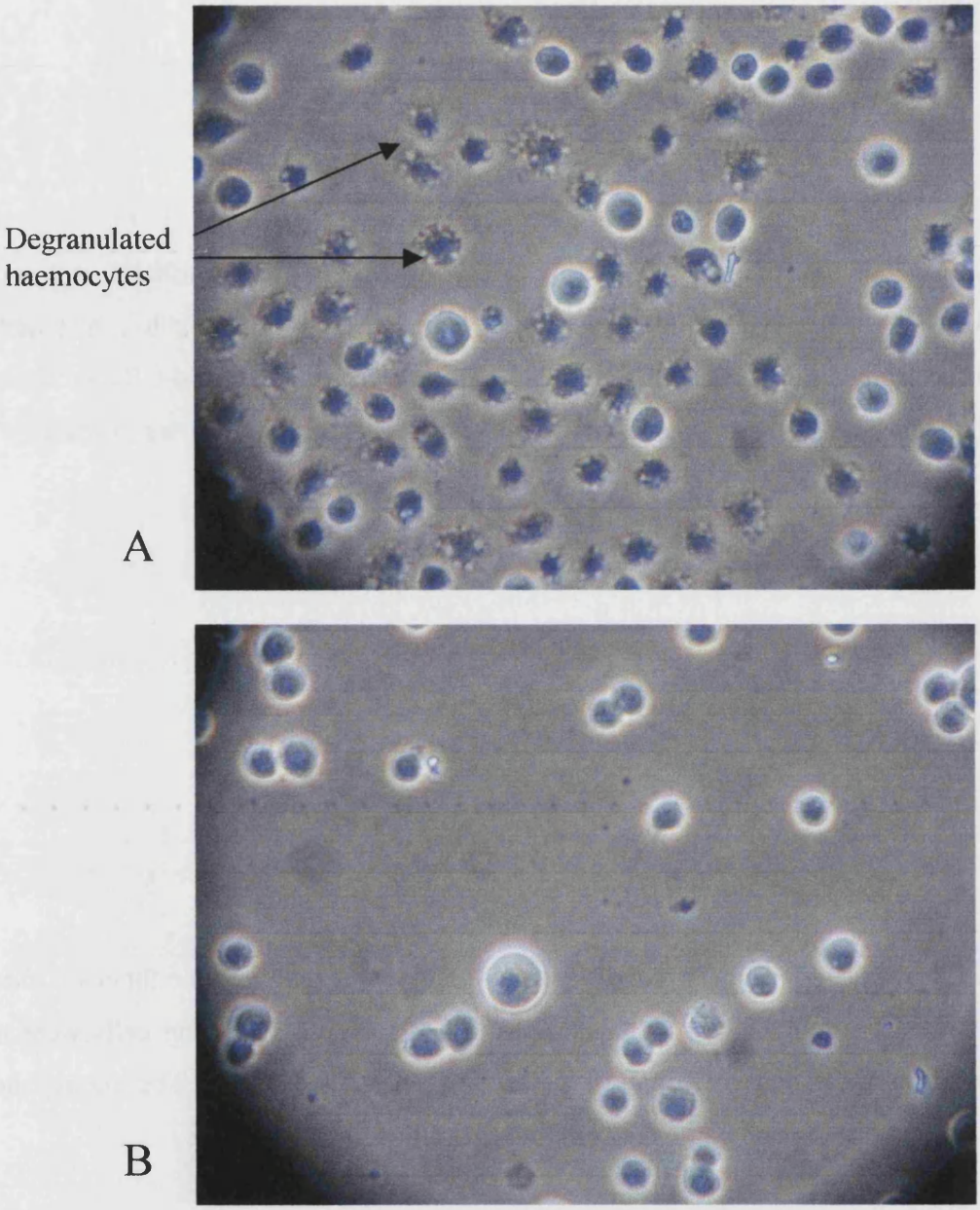
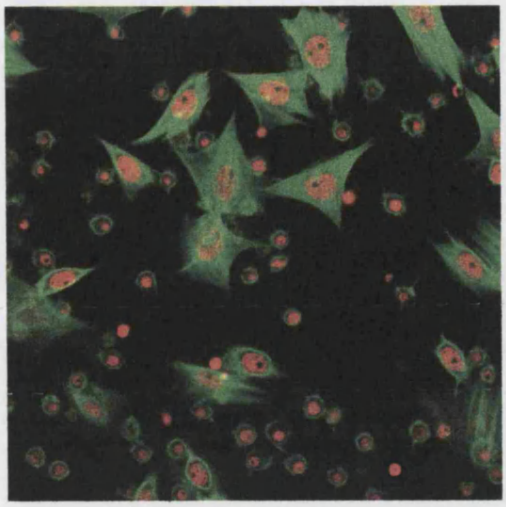


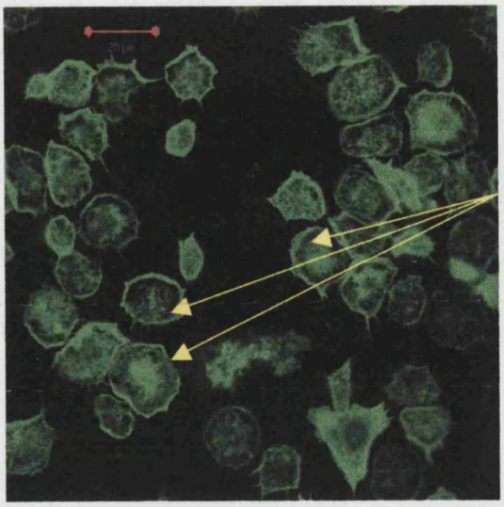
Fig. 11A and B. Phase-contrast micrographs monolayers of haemocyte exposed to laminarin *in vivo* (A) and *in vitro* (B). Haemocytes from larvae injected with laminarin at 17 h post-injection (Fig. A) displayed much degranulation (note vacuoles) and appeared phase-dark. Haemocytes in Fig. B were exposed to 0.05% laminarin *in vitro* for 24 h and no degranulation was observed.

Fig. 11C. Monolayers from larvae injected with a solution of laminarin at 3 and 17 h post-injection. Red = propidium iodide (nucleus), green = FITC-phalloidin (F-actin). At 17 h post-injection, most of the granular cells have degranulated (d-GR) and compared with the GR at 3 h, have spread and appeared vacuolar. Both figures are to scale.

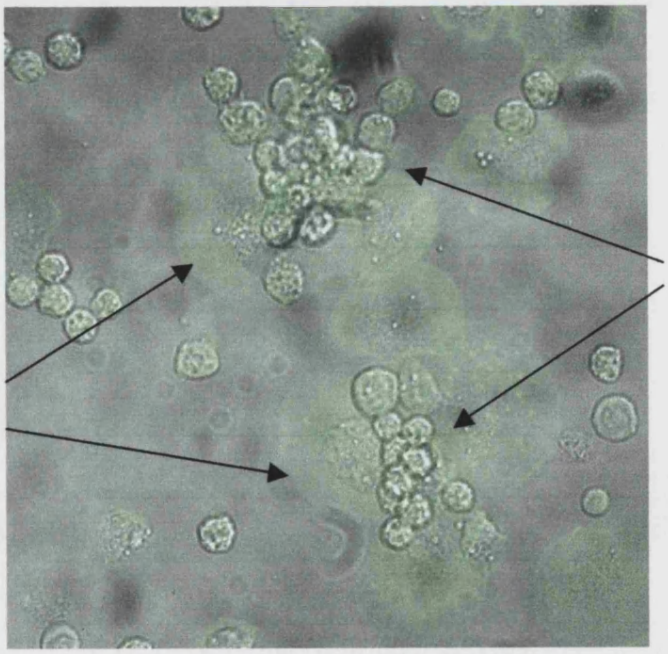
Fig. 12. Microaggregates in monolayers from larvae infected with the fungus *B. bassiana* 304 were invariably found associated with VLBC. These spreading cells were almost impossible to visualise without the use of phalloidin staining. The figure shows a transmitted phase-contrast image combined with fluorescence image.



3 h



17 h



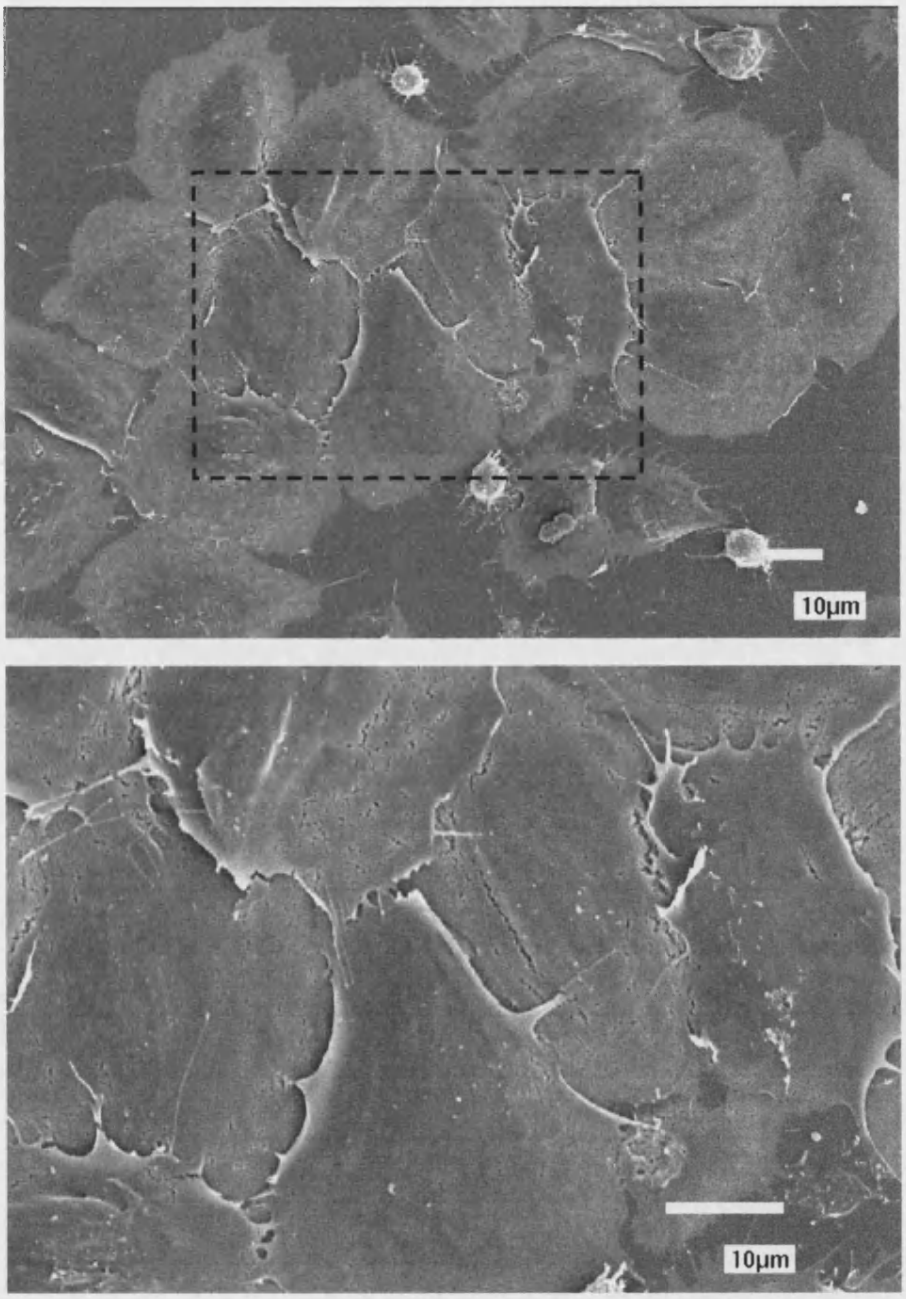


Fig. 13. Scanning electron micrograph shows VLBC of the 'fried egg' type have an affinity for one another and form confluent, flat layers on the coverslip. The dashed box in the upper figure is magnified in the lower figure and shows that these cell types could form a very effective barrier around nodules/capsules.

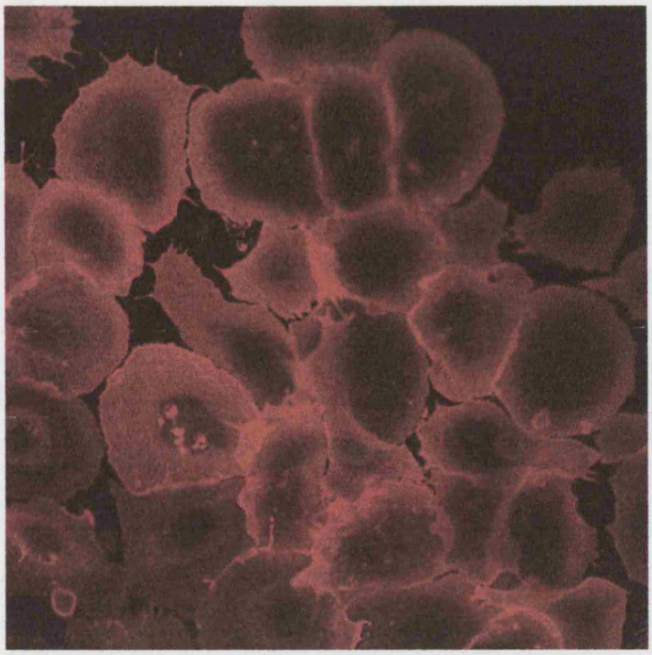


Fig. 14. Hyper-spreading cells label strongly with the monoclonal antibody MS13. This antibody is known to specifically label conventional plasmatocytes. The VLBC types in this figure are of the fried egg morphology although the larger 'speckled' VLBC type also labelled with the antibody. The figure shows a confocal image at x 630 magnification.

5.3 Discussion

This study describes a novel immune response to bacterial and fungal infections in *Manduca sexta*. Bacterial and fungal infection and the injection of a laminarin in this insect caused the appearance of haemocytes in monolayers with an extreme spreading ability. I am unaware of previous studies that report an induction of haemocyte spreading by microbial infections. Very large blood cells (VLBC) appear large *in vitro* because of their extreme spreading ability on a glass surface. They are not large *in vivo*. The appearance of hyperspread haemocytes in response to a variety of microorganisms and microbial antigens is evidence for a general immune response in *M. sexta* to microbial infection.

Most healthy larvae possessed no VLBC as haemocyte monolayers from healthy larvae consisted mainly of plasmatocytes (PL) and granular cells (GR). As with other insects, *M. sexta* PL are characterised by, in part, their ability to spread *in vitro* (Willot *et al.*, 1994), but the degree of spreading displayed by conventional PL in the present study was significantly less than the VLBC. By day-2 post-infection with *B. bassiana*, there was a dramatic change in the appearance of the monolayers as much of the area of coverslip was taken up by large spreading haemocytes. This effect is unlikely to be caused directly by the fungus because at the time VLBC appear, the level of the fungus *in vivo* was small and any pathological (e.g. toxicological) effects on the haemocytes are unlikely. Moreover, the non-pathogenic *E. coli* (which was heat-killed and should not be toxic to the larvae) also induced the appearance of VLBC suggesting the response is general and is indeed initiated by the insect.

The origin of VLBC is unclear but there are 2 possibilities:

(1) VLBC are regular haemocytes (i.e. plasmatocytes) that differentiate or become 'primed' upon infection with the ability to spread extensively. In *M. sexta*, both PL and VLBC were consistently present at the same time in infected larvae, thus if VLBC are differentiated PL then not all PL are involved in this transformation. Willot *et al.* (2002) showed that regular PL have the ability to spread to a large diameter *in vitro* and that this correlated with Ca^{2+} concentration. They also report that a few PL were seen to spread extensively and assumed a 'fried egg' shape. This morphology is consistent with my description of the morphology

of the 'fried egg' VLBC type. However, Willot *et al.* (2002) reported that most spread PL did not have a fried egg shape but were elongate when well spread, typical of many PL. This group did not describe the larger 'speckled' VLBC type in their spreading assays.

VLBC were labelled with the monoclonal antibody MS13, known to serve as a marker for PL (Willot *et al.*, 2000). Based on this result it cannot be assumed that VLBC are well-spread PL but only that they share a particular cell surface epitope. Interestingly, MS13 recognises a haemocyte membrane protein important in cell spreading (Willot *et al.*, 2000) so it is not surprising that VLBC label strongly with this antibody.

(2) VLBC are new cell types that are released into circulation upon infection. This may occur either by the mobilisation of sessile pools of VLBC or be the result of stem cell differentiation in haemopoietic organs. In *Drosophila melanogaster*, a new cell type, the lamellocyte are seldom found in non-immune larvae but appear upon parasitisation. Interestingly, lamellocytes are characterised by their large and flat morphology suggesting possible functional similarities with VLBC (discussed later). The lamellocytes are released from the *Drosophila* lymph gland or haemopoietic organ, which apparently detects the presence of the parasitoid within the haemocoel (Lanot *et al.*, 2001; Sorrentino *et al.*, 2002). After immune challenge (by injection of a large foreign object or parasitization), the cells of the lymph gland undergo massive cell division and differentiation into new cells, the lamellocytes, which are released into the haemolymph (Lanot *et al.*, 2001; Sorrentino *et al.*, 2002).

If VLBC were derived from existing haemocytes then we would expect some kind of signalling factor in the plasma. I attempted to induce VLBC from haemocytes in healthy monolayers using plasma from larvae at different stages of fungal infection. VLBC could not be induced by this method. I also assessed the spreading of haemocytes in the presence of fungal filtrate, fungal conidia and laminarin and spreading was not increased under these conditions. Although efforts were largely unsuccessful, it still cannot be ruled out that VLBC are well-spread or differentiated plasmatocytes that have been induced by a signal *in vivo*. If a spreading-stimulating factor is present in the plasma upon infection then it may be very short-lived *in vivo* or unstable *in vitro* to observe its effects on the plasmatocytes. Many factors that affect plasmatocyte spreading have been reported in the literature

including soluble peptides containing the amino acid sequence RGD (Pech and Strand, 1995), ENF peptides which include the plasmatocyte spreading peptide from *Pseudoplusia* includens (Strand *et al.*, 2000), calcium levels (Willot *et al.*, 2002) and eicosanoids (Mandato *et al.*, 1997). Thus, there is high potential for the presence of a spreading factor in the haemolymph upon infection.

As the fungal infection progressed, the number of VLBC in the monolayer declined and by day 4 post-infection, no VLBC were present in any of the infected larvae. It is possible that VLBC are used up by the immune system i.e. taken out of circulation by processes such as nodule formation and then not replaced. Microaggregates (incipient nodules) from fungal-infected larvae were invariably associated with VLBC in the monolayers suggesting these cells take part in nodulation. It is well established that plasmatocytes involved in nodule formation spread extensively to form multiple overlapping layers which aid in the trapping and isolation of invaders (Lackie, 1988). The lamellocytes perform this function in *D. melanogaster* during encapsulation of parasitoids eggs (for a review see Carton and Nappi, 1997) and it seems likely that VLBC in *M. sexta* may have a similar role in nodule formation. Like lamellocytes and encapsulation, VLBC would be remarkably effective in this role in nodule formation, with the ability to spread quickly and form very thin layers around the core of the early nodule. The reason VLBC spread extensively upon contact with the glass surface is unclear but it may be that the VLBC recognise the glass surface as they would a nodule or capsule and respond by spreading. I performed some SEM to show that VLBC do have a tendency to clump together on the monolayer and in doing so form confluent layers, typical of flattening haemocytes associated with nodule formation.

VLBC did not appear in monolayers upon parasitization with wasps or nematodes although a complete time course was not performed so results are inconclusive. Furthermore, immune suppression by these insect parasites may include destruction of haemocyte types such as VLBC. As these parasitoids are likely to suppress or avoid cellular immune responses like encapsulation, a role for VLBC in encapsulation cannot be discounted based on the findings of the present study and more work is required here.

Another possibility why VLBC numbers decline during fungal infection is due to a pathological effect of the fungus. *Beauveria bassiana,* as with many other

entomopathogenic fungi, produce a variety of metabolites *in vitro* that actively suppress the immune system (Griesch and Vilcinskas, 1998). It was observed in the present study that fungal-infected plasma and fungal culture filtrate both had a suppressive effect on the spreading of haemocytes in monolayers from control and infected larvae and, in most cases the haemocytes detached from the glass surface. This suggests this fungus produces anti-spreading factors and it has been established previously that proteases produced by *B. bassiana* have a suppressive effect on haemocyte spreading (Griesch and Vilcinskas, 1998), though their role in infection is less established. In the beet armyworm, *Spodoptera exigua*, Hung *et al.* (1993) have shown that infection with *B. bassiana* results in a reduced spreading ability of this insect's haemocytes. A later study by the same group showed that toxic metabolites isolated from *B. bassiana*-infected haemolymph also suppressed haemocyte spreading when injected into healthy larvae (Mazet *et al.*, 1994). Thus, the loss in the VLBC morphology during late-stage *B. bassiana* infection may be due to immunosuppressive metabolites from the fungus.

VLBC may also be selectively killed by the fungus. Selective killing of haemocyte types (i.e. lamellocytes) by injected virus-like particles has been shown to occur in *D. melanogaster* upon parasitisation by the endoparasitic wasp, *Leptopilina heterotoma* (Rizki and Rizki, 1984b; Rizki and Rizki, 1990). The killing of the lamellocytes by the parasitoid abrogates the encapsulation response of *D. melanogaster* and allows for normal development of the parasitoid larvae *in vivo*.

Laminarin stimulated the appearance of VLBC. Laminarin is comprised largely of ß-1,3-glucans, a fungal polymer that is present in fungal cell walls. VLBC first appeared in the haemolymph at 24 h post-injection with laminarin. A 24 h delay of VLBC induction was also observed with the injection of *E. coli* and a 30 h delay with the injection *P. luminescens*. As the response is not immediate, it seems likely that events such as signal transduction and gene transcription is occurring leading to the induction of VLBC. In addition, at 12 h post-injection with laminarin, the protein profile of the haemolymph changed and at 17 h most of the granular cells had degranulated. These events preceded the induction of VLBC and it is possible they are related. The contents of GR that are released into the plasma upon degranulation may be important in the induction of VLBC. Laminarin did not, however, induce VLBC *in vitro* in healthy monolayers nor did it cause a

degranulation of GR as seen *in vivo*. Given that GR did not degranulate when exposed directly to laminarin suggests another factor(s) was responsible for the effects *in vivo*.

The decline of VLBC on successive days after 24 h in laminarin-injected larvae was not as pronounced as that seen in larvae infected with the fungus, supporting the idea that the fungus may be actively affecting the spreading response. Although laminarin is abiotic, it did cause larvae to melanise and slowed their growth rate so it cannot be assumed non-toxic at the concentration used. The decline in VLBC observed with the laminarin-injected larvae may be due to VLBC being removed from circulation in nodule formation (as laminarin stimulated the production of many microaggregates in the present study) or may be due to a decline in fitness of the immune system.

Due to time constraints, it was not possible to characterise VLBC more completely. A more complete time course with *E. coli* (heat-killed) would reveal if the decline of VLBC during laminarin and fungal infection is toxicity related. Furthermore, a more detailed time course of the haemocyte profile in nematode and wasp-parasitized larvae and injection of foreign objects that promote encapsulation would be necessary to discover whether VLBC are important in encapsulation.

The spreading response of haemocytes to infection reported in this study has not been reported previously in *M. sexta* or any other insect. The preparation of the monolayer may be a vital factor in the observation of these cells. It has been shown that in *M. sexta*, the presence of EDTA (ethylenediaminetetraacetic acid), which is a common component of anticoagulant saline, strongly suppresses haemocyte spreading (Willott *et al.*, 2002) and may have similar effects on VLBC. I did not use EDTA but note that many researchers commonly use this reagent in monolayer preparation. It is also possible that due to their extreme thinness, VLBC-like cells are not easily noticeable without the use of specific staining or electron microscopy. In addition, as shown with the fungal infection, VLBC appear to be transient and disappear from the haemolymph during fungal infection, thus they could be easily overlooked.

Chapter 6

Modulation by eicosanoid biosynthesis inhibitors of immune responses in the insect *Manduca sexta* to the pathogenic fungus *Metarhizium anisopliae*

***A slightly modified version of this chapter has been published in Journal of Invertebrate Pathology volume 79 pp 93-101.**

6.1 Introduction

Eicosanoids are biologically active, oxygenated metabolites of arachidonic acid (AA) and two other C20 polyunsaturated fatty acids (Corey *et al.*, 1980). They are formed when AA is liberated from membrane phospholipids through the action of phospholipase A_2 (PLA_2). This free AA is then metabolised down three different biosynthetic routes giving rise to three eicosanoid families: (1) prostaglandins and thromboxanes, formed *via* the cycloxygenase pathway; (2) leukotrienes, lipoxins and other products of the lipoxygenase pathway; (3) epoxyeicosatrienoic acids of the cytochrome P_{450} (or epoxygenase) pathway (Stanley-Samuelson, 1994).

Eicosanoids have been implicated in the functioning of the insect immune system, especially in nodule formation, the major cellular immune response to bacterial infections (Horohov and Dunn, 1983; Ratcliffe and Walters, 1983). Nodule formation occurs very quickly after immune challenge and involves haemocyte aggregation, during which a multicellular structure or nodule is formed (Ratcliffe and Rowley, 1979). During the process, a large number of bacteria become entrapped within the nodule, essentially isolating them from the insect haemocoel (see general introduction for a detailed description).

Stanley-Samuelson *et al.* (1991) first showed that compounds such as dexamethasone, that are well known eicosanoid biosynthesis inhibitors in vertebrates, reduced the ability of the tobacco hornworm *Manduca sexta* to clear injected bacteria from the haemolymph

and thus enhanced larval mortality caused by the septicaemia. Miller *et al.* (1994) expanded on these findings to show that the impaired immune function caused by these inhibitors in *M. sexta* was correlated with an inhibition of nodule formation. The physiological relevance of these results has been strengthened by the findings that both eicosanoids and the enzymes that synthesise them are present in *M. sexta* tissues (Stanley-Samuelson *et al.*, 1991). Of particular interest was the discovery that various eicosanoids are biosynthesised in the fat body (Stanley-Samuelson and Ogg, 1994) and haemocytes (Gadelhak *et al.*, 1995) of *M. sexta,* two tissues that have a central role in the insect immune response. The formation of these compounds was inhibited by eicosanoid biosynthesis inhibitors. Most recently, Miller and Stanley (2001) have shown that eicosanoid biosynthesis inhibitors have a direct action on *M. sexta* haemocytes themselves.

Several other insect species, from many different Orders have been studied in an attempt to generalise the finding that eicosanoids mediate nodule formation during infection in insects (Miller *et al.*, 1996; Jurenka *et al.*, 1997; Miller *et al.*, 1999; Stanley *et al.*, 1999; Stanley-Samuelson *et al.*, 1997). However, almost all previous work has been concerned with immune responses provoked by bacteria. The study of Mandato *et al.* (1997) is an exception in using silica microspheres, while Bedick *et al.* (2000) used the bacterial cell wall component, lipopolysaccharide (LPS) to elicit nodule formation. Recently, Carton *et al.* (2002) suggested that encapsulation (a similar response to nodulation) of parasitoid eggs in the fruit fly *Drosophila melanogaster*, is also mediated by eicosanoids.

To date, there appear to be no studies that have examined the possible role of eicosanoids in modulating insect immune responses to entomopathogenic fungi. This question is important because entomopathogenic fungi are an important challenge to the insect immune system under natural conditions, frequently acting as major regulators of insect populations (Hajek, 1997). Moreover, it has been shown that at least in *D. melanogaster*, the insect immune system discriminates between bacteria and fungi, activating different signal transduction pathways according to the kind of microorganism detected (Khush *et al.*, 2001; Lemaitre *et al.*, 1997;).

In this study, I have used the deuteromycete *Metarhizium anisopliae* as a model entomopathogenic fungus. *M. anisopliae* occurs worldwide and infects a wide range

of host insects (Tanada and Kaya, 1993). I report here the effects of eicosanoid biosynthesis inhibitors on the *M. sexta* immune response toward *M. anisopliae*. In addition, evidence is presented that larvae treated with these inhibitors become more susceptible to fungal infection and that this immune impairment is correlated with the suppression of nodule formation.

6.2 Results

6.2.1 Effect of dexamethasone on the mortality of larvae infected with fungus

Different doses of *M.anisopliae* ME1 conidia were injected into the haemocoel of *M. sexta* larvae to find a suitable dose for the mortality assay. Host mortality was dependent on conidial dose with increasing dose causing earlier mortality (Fig. 1). An injected dose of 4 X 10^3 conidia was sufficient to kill the insects over a period that was suitable for testing the effects of dexamethasone.

Dexamethasone strongly enhanced the speed of kill of the insect by the fungus (Fig. 2, χ^2 test at time point when control mortality had reached 40%, $P < 0.001$). This effect was significantly reversed when AA was co-injected with the dexamethasone as there was no significant difference between the mortality of control larvae (ethanol-injected) and dexamethasone/AA injected larvae (χ^2 test, $P > 0.1$).

6.2.2 Effect of eicosanoid biosynthesis inhibitors on weight gain of larvae infected with the fungus

Reduction in weight gain is a common symptom of fungal infection in insects and this was true for *M. sexta* infected with *M. anisopliae* (Fig. 3). There was a negative relationship between fungal dose and increase in weight. All insects underwent an initial slight decrease in weight due to removal from diet over the first 24 hours. High doses of conidia caused mortality of the larvae early in the experiment and, for this reason, a complete time course could not be achieved for these doses.

Weight gain of infected larvae injected with various eicosanoid biosynthesis inhibitors was taken on day 3 post-injection. All eicosanoid biosynthesis inhibitors caused a

significant decrease in weight gain in the infected larvae (Fig. 4A). Thus, in each case, weight gain of *M. anisopliae*-infected larvae injected with indomethacin, ibuprofen, phenidone, esculetin or dexamethasone was significantly less than the corresponding weight gain of the control (ethanol-injected). Injection of any of these inhibitors alone (without conidia) had no effects on the growth rate of the larvae relative to controls (Fig. 4B).

The effect of dexamethasone on the weight gain of infected larvae was determined on a daily basis (Fig. 5), and was found to cause a significant reduction in weight gain as early as day 2 post-injection ($P < 0.01$). This effect became more significant on successive days. Injecting dexamethasone alone into larvae that had received no conidia had no effect on the weight gain of the larvae, compared with the uninjected controls.

6.2.3 Effect of dexamethasone on nodule formation *in vivo*

Nodule formation in *M. sexta* was found to be an important part of the defensive response toward *M. anisopliae*. A large number of nodules (108 nodules) was formed *in vivo* in response to injection of 30, 000 *M. anisopliae* conidia (Fig. 6). When dexamethasone was co-injected with *M.anisopliae* conidia, the number of nodules formed was strongly reduced (21 nodules) compared with the conidia alone (Mann-Whitney U-test, $P < 0.001$). The suppressive effect of dexamethasone was significantly reversed ($P < 0.001$) by the co-administration of AA with dexamethasone (83 nodules). Negligible numbers of nodules were formed when larvae were treated with AA alone, without conidia.

6.2.4 Effect of dexamethasone on haemocyte microaggregate formation *in vitro*

Dexamethasone also reduced the formation of haemocyte microaggregates *in vitro* (Fig. 7, $P < 0.05$), suggesting that the action of this eicosanoid biosynthesis inhibitor on haemocytes is direct. The toxicity assay shown in Fig. 8 reveals that the concentration of dexamethasone used in the assay did not affect the viability of haemocytes. The addition of AA appeared to reduce the inhibitory effect of dexamethasone but the number of microaggregates formed was not significantly enhanced over the level seen with dexamethasone alone ($P = 0.26$). On the other hand, the number of microaggregates formed with the combined AA/dexamethasone treatment was not significantly different

to the control level (P = 0.12), unlike the case when dexamethasone was given alone. Although a background level of microaggregate formation was observed with unchallenged haemocytes, the number of microaggregates formed was very small compared with the response to conidia. AA alone did not induce the formation of microaggregates above the background level.

6.2.5 Effect of dexamethasone and ibuprofen on phagocytosis and cell spreading

Dexamethasone and ibuprofen had no effect on the ability of *M. sexta* haemocytes to phagocytose *E. coli in vitro* (Table 1). Haemocyte monolayers were prepared and incubated with the bacteria in the presence of the inhibitors. There was no significant difference between the levels of phagocytosis displayed by the control monolayers (ethanol-exposed) and those exposed to the inhibitors (Kruskal-Wallis, P = 0.38). Furthermore, these drugs had no visible effect on haemocyte spreading and overall haemocyte morphology as assessed by confocal microscopy (results not shown).

6.2.6 Toxicity assays

Data for the *in vitro* toxicity assays of dexamethasone and AA are given in Fig. 8. Toxicity was detected by the percentage of haemocytes that took up the viability stain, trypan blue. Dexamethasone was non-toxic at all concentrations tested, even at 540 μM (over 6 times the concentration actually used in the *in vitro* microaggregation assay). Therefore, the inhibitory effect of dexamethasone on microaggregate formation *in vitro* was not simply due to toxicity. By contrast, AA caused significant haemocyte mortality at concentrations of 5.4 μM and above. Thus, for this reason, a concentration of 2.7 μM AA was used the *in vitro* nodulation assay.

6.2.7 Effects of dexamethasone on germination and hyphal growth of the fungus

To test whether dexamethasone had any direct effects on the fungus, conidial germination and hyphal elongation were assessed *in vitro* in the presence of this inhibitor. Compared to the controls (ethanol-exposed), percentage germination of *M. anisopliae* on media containing dexamethasone (85 μM final concentration) was not significantly different (Ethanol, 95.5 ± 3.5%; Dexamethasone, 96.34 ± 2.7%; means ± SD; t-test P = 0.76, n

= 1000 conidia). In addition, growth of the emergent hyphae *in vitro* was unaffected by the dexamethasone compared to controls (Ethanol: 7.4 ± 3.3 arbitrary units; Dexamethasone: 7.4 ± 3.0 arbitrary units; mean lengths ± SD [t-test, P = 0.68; n = 250 hyphae counted per treatment]).

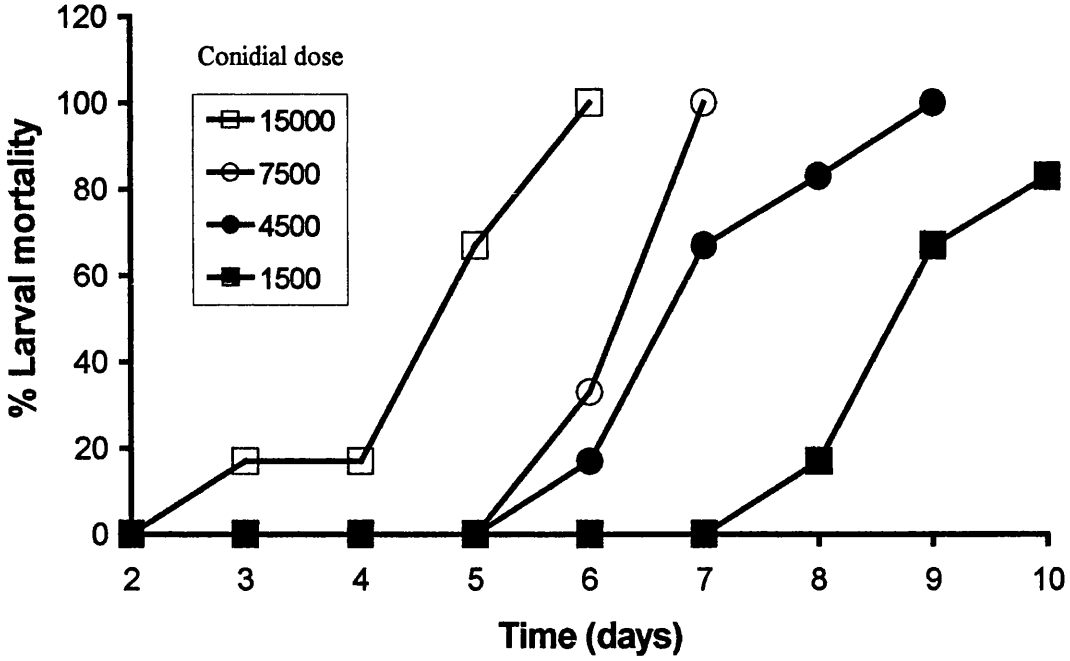
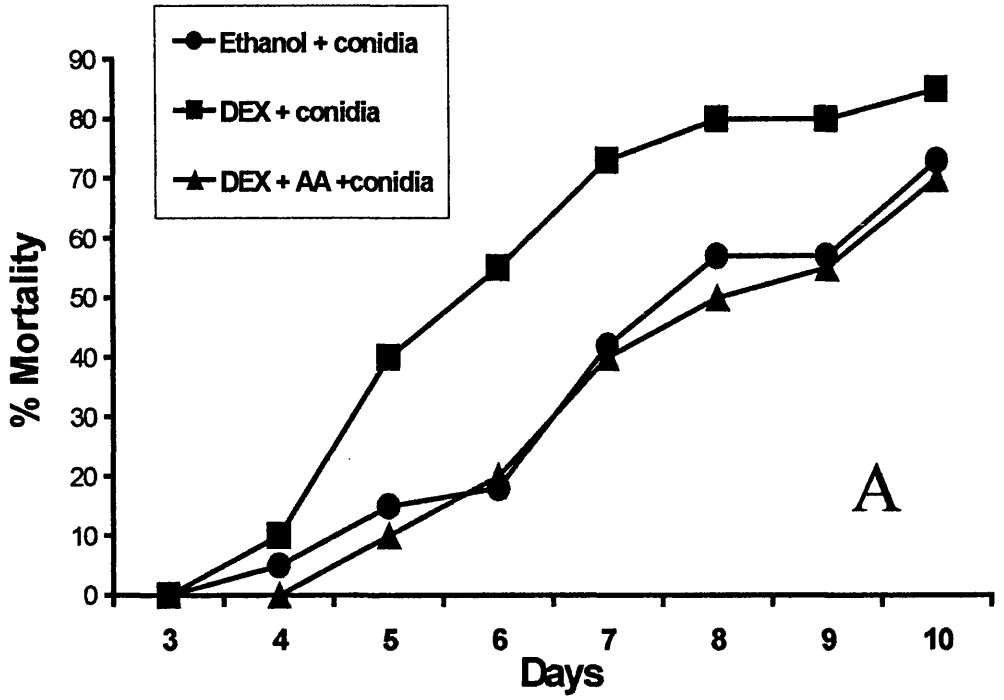
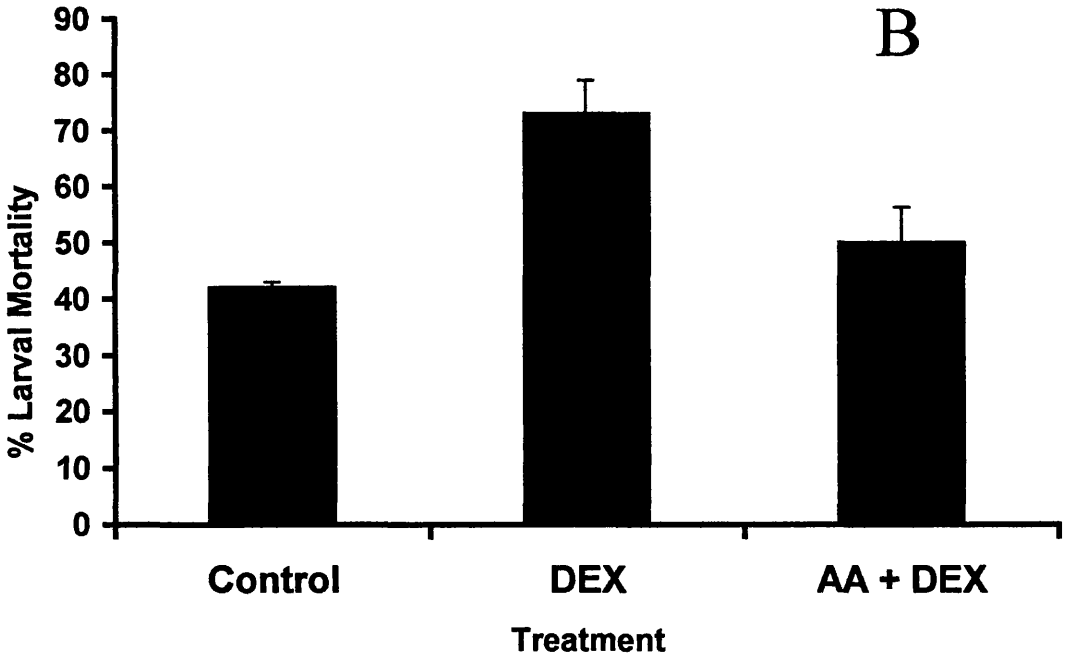


Fig. 1. Progressive mortality of *Manduca sexta* caused by the pathogenic fungus *Metarhizium anisopliae*. Fifth instar larvae were selected on the first day after moulting (day 0) when they were injected with 15 µl of a suspension of *M. anisopliae* conidia (spores) in 0.05% Tween 80, containing the indicated number of conidia. The points show percentage mortality on days post-inoculation (6 larvae for each concentration of conidia). Control larvae (injected with 0.05% Tween 80) showed no mortality (results not shown).


Ethanol + conidia
DEX + conidia
DEX + AA +conidia
% Mortality
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
10
Days
A


B
% Larval Mortality
90
80
70
60
50
40
30
20
10
0
Control
DEX
AA + DEX
Treatment

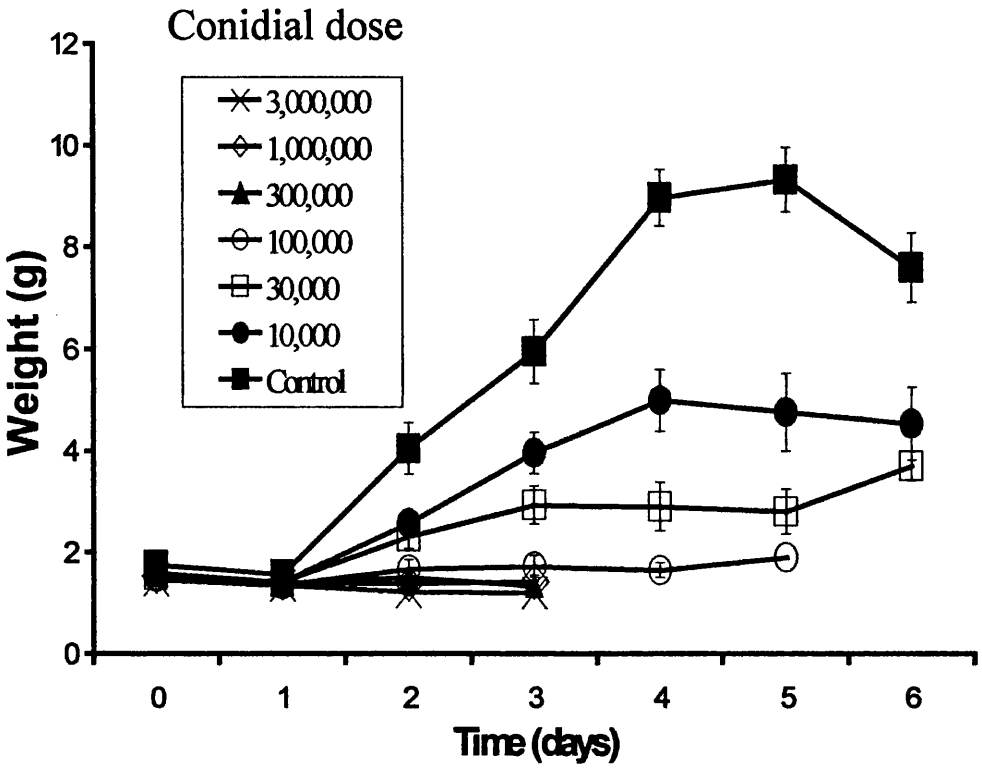


Fig. 3. Effect of *M. anisopliae* mycosis on weight gain of fifth instar *M. sexta* larvae. Suspensions of fungal conidia were injected as described in the legend to Fig. 1. The total number of conidia injected per insect is shown in each case. Controls received 0.05% Tween 80 without conidia. The larvae were weighed daily. Where no point is shown, the insects were dead due to mycosis. Means $\pm$ S.E. (n = 10).

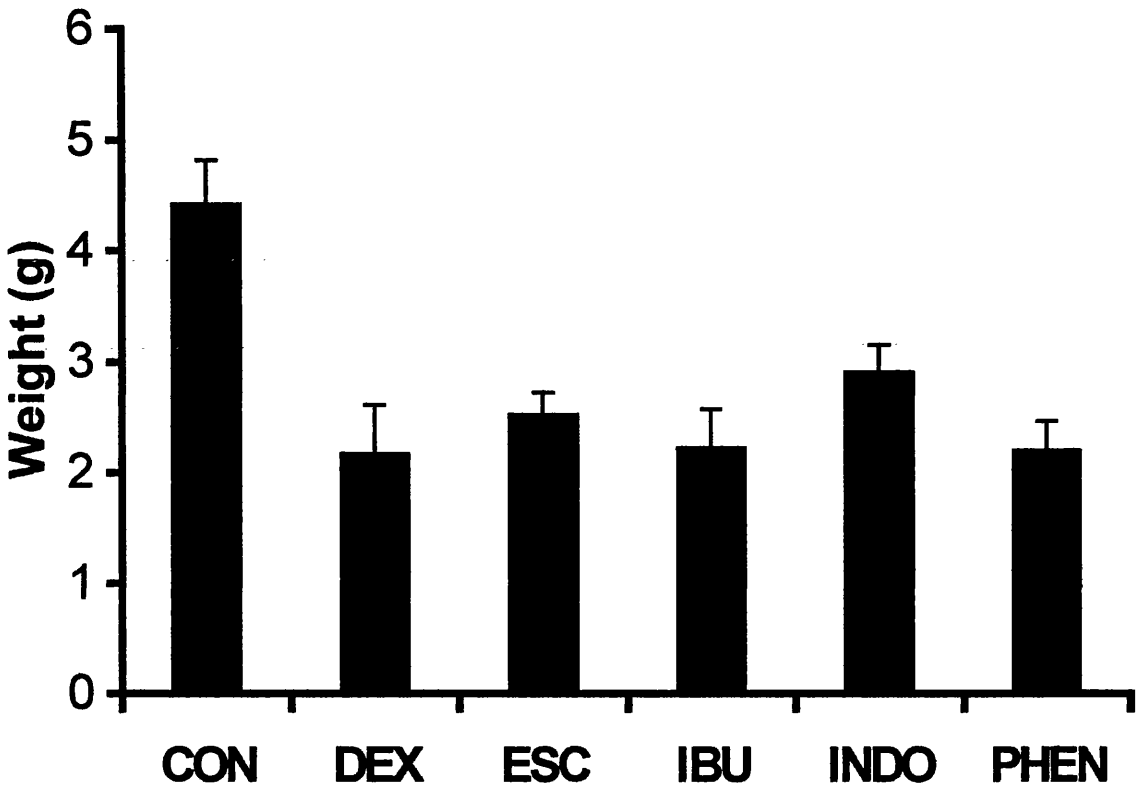


Fig. 4A. Effect of eicosanoid biosynthesis inhibitors on weight gain of *M. sexta* larvae infected with *M. anisopliae*. Day 0 fifth instar larvae were injected with 10 μg inhibitor or 95% ethanol (control) and 15 min later were injected (15 μl) with a suspension containing 30, 000 *M. anisopliae* conidia. Weight gain was measured from day 0-3 post-treatment and the mean ± S.E. are shown for each treatment (n = 5). All eicosanoid biosynthesis inhibitors significantly decreased weight gain relative to controls (Mann-Whitney U-test, $P < 0.05$). CON = control, DEX = dexamethasone, ESC = esculetin, IBU = ibuprofen, INDO = indomethacin, PHEN = phenidone.

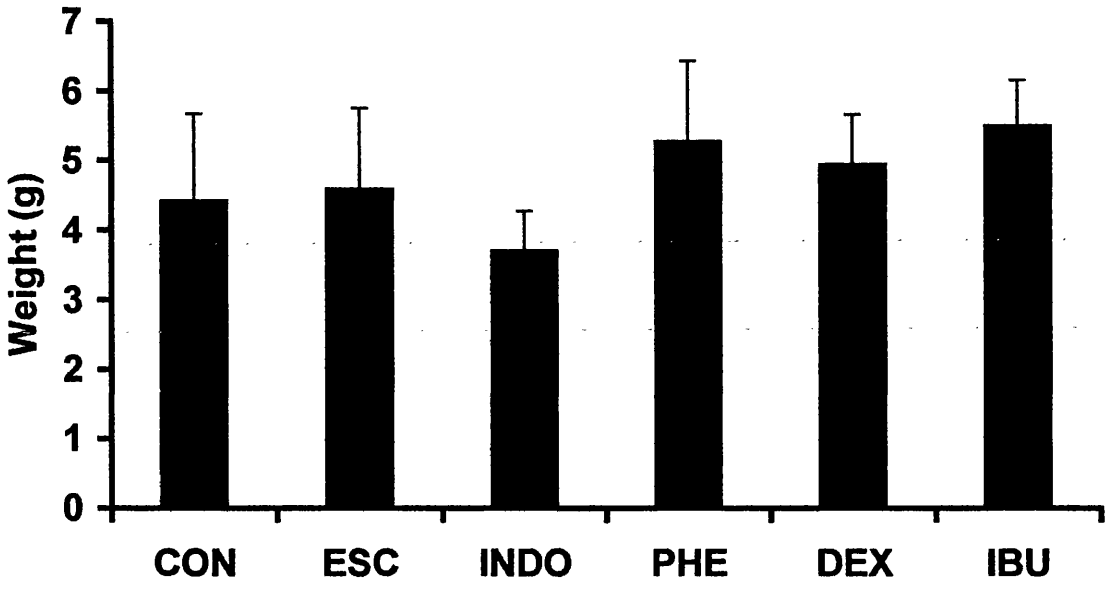


Fig. 4B. Effect of eicosanoid biosynthesis inhibitors on weight gain of non-infected *M. sexta* larvae. Day 0 fifth instar larvae were injected with 10 μg inhibitor or 95% ethanol (control) and weight gain was measured from day 0-3 post-treatment. Mean ± S.E. are shown for each treatment (n = 5). There was no significant difference between the weight gain of control larvae and those injected with the inhibitors ($P > 0.1$ in each each, Mann-Whitney U-test). CON = control, DEX = dexamethasone, ESC = esculetin, IBU = ibuprofen, INDO = indomethacin, PHEN = phenidone.

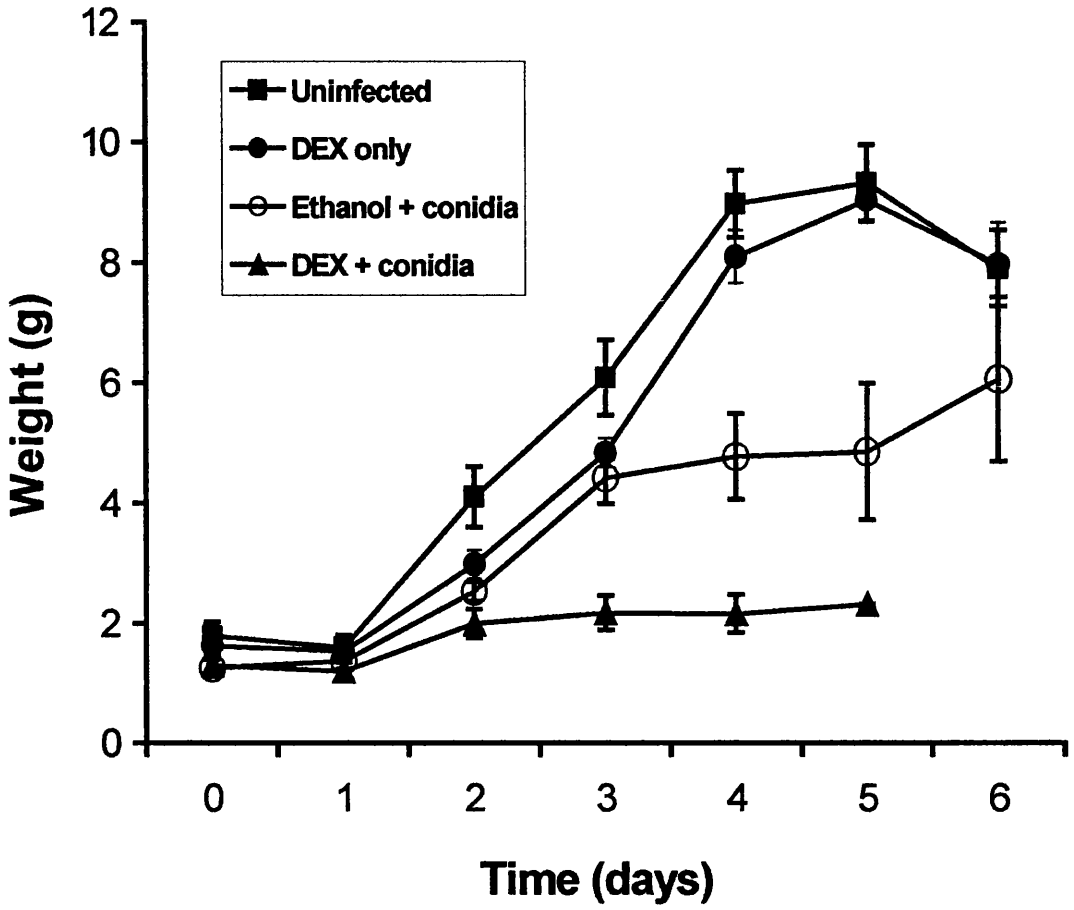


Fig. 5. Effect of dexamethasone (DEX) on weight gain of *M. sexta* infected with *M. anisopliae*. Day 0 fifth instar larvae were injected with dexamethasone or solvent alone (ethanol). 15 min later, all larvae (except the "uninfected" and "DEX only" group) were injected (15 µl) with a suspension containing 30, 000 *M. anisopliae* conidia in 0.05% Tween 80. "Uninfected" larvae were injected with 15 µl 0.05% Tween 80 without conidia. Each point represents the mean ± S.E. (n = 5, uninfected = 10).

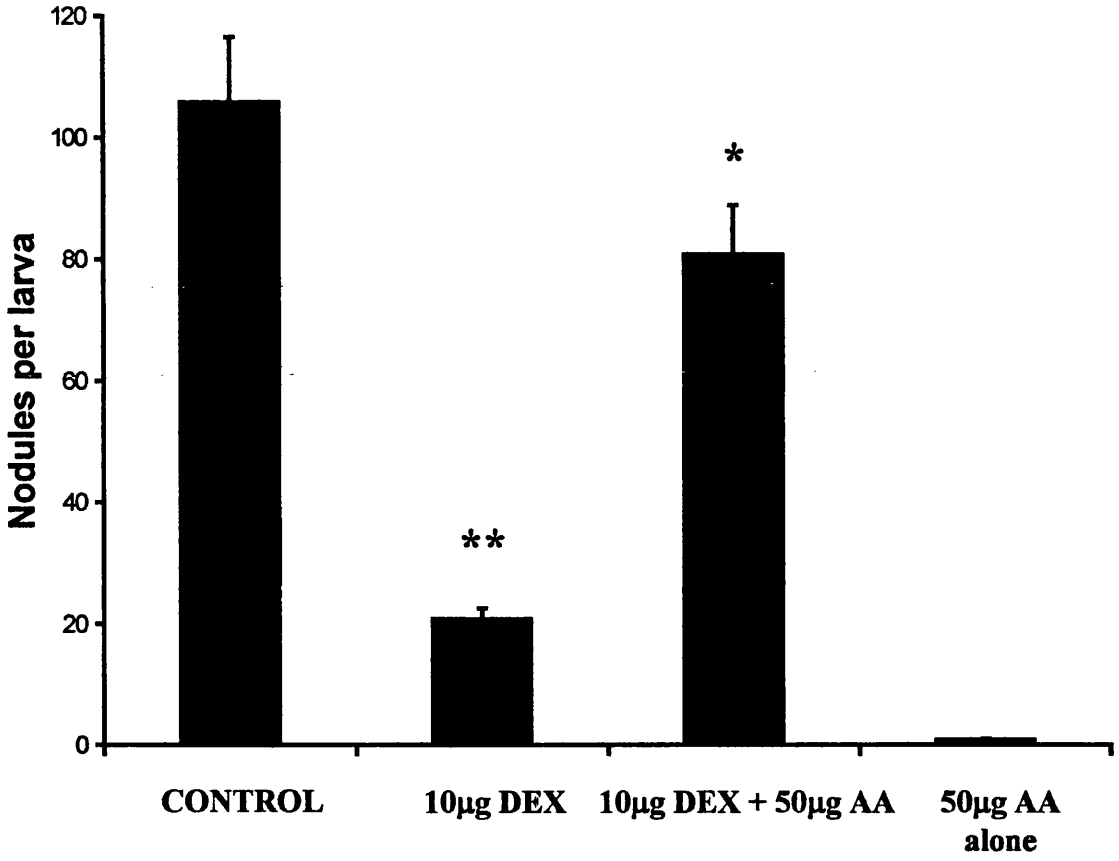


Fig. 6. Effect of dexamethasone (DEX) and arachidonic acid (AA) on nodule formation *in vivo*. Day 0 fifth instar larvae of *M. sexta* were injected with the indicated substances. Control larvae received solvent (ethanol) alone. 15 min later they were injected (15 µl) with 30, 000 *M. anisopliae* conidia suspended in 15 µl 0.05% Tween 80. After 24 h the larvae were dissected and nodules counted. For each treatment, the height of the bars show the mean number of nodules ± S.E. (n = 12). Significant difference, relative to control is indicated by asterisks: * $P < 0.05$; ** $P < 0.001$.

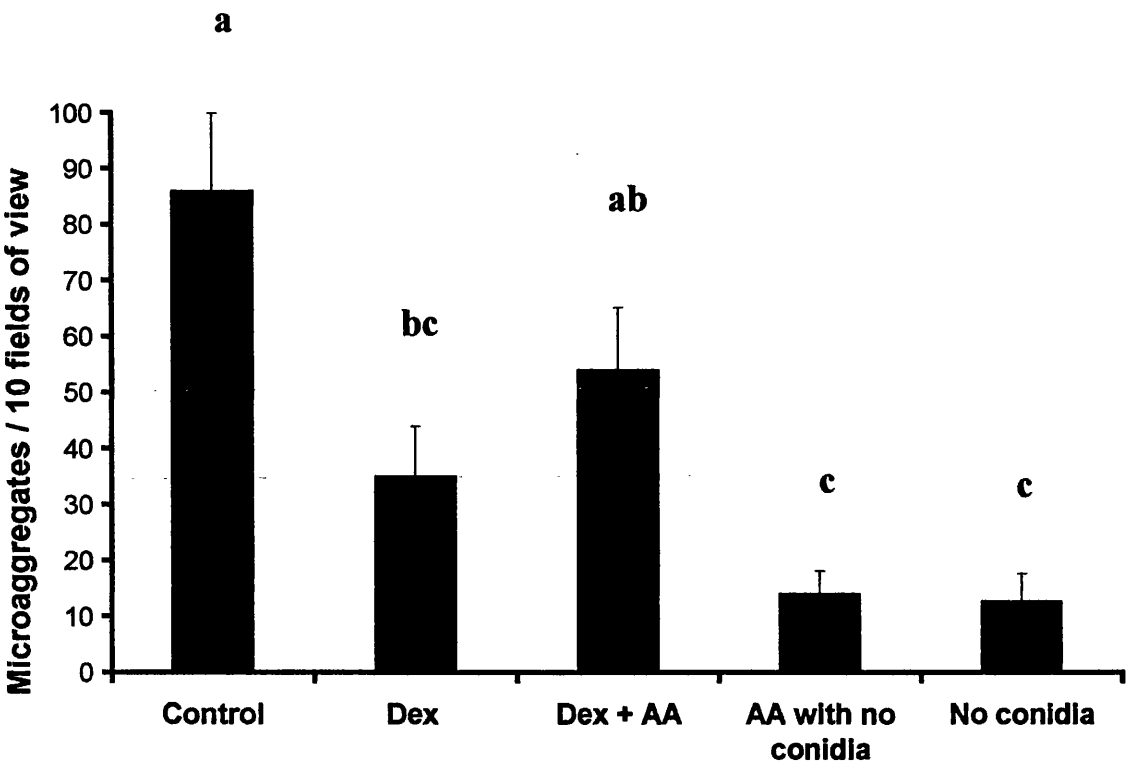


Fig. 7. Effect of dexamethasone (DEX) and arachidonic acid (AA) on haemocyte microaggregation *in vitro*. The protocol used is described in the text. For each treatment, the height of the bars shows the number of microaggregates formed (mean $\pm$ S.E.; n = 12). Significant differences between treatments are indicated by different letters (Mann-Whitney U-test, $P < 0.05$).

	TREATMENT		
% Phagocytosis	Ethanol alone	Ibuprofen (50 μM)	Dexamethasone (50 μM)
Mean	61.92	57.22	61.7
SE	2.2	3.56	3.9

Table 1. The effect of dexamethasone and ibuprofen on the phagocytosis of *E. coli* by *M. sexta* haemocytes *in vitro*. Haemocyte monolayers were exposed to the different eicosanoid biosynthesis inhibitors at the concentrations shown. Control monolayers were exposed to ethanol alone. The cells were then incubated with FITC-labelled *E. coli* for 2 h and the resulting level of phagocytosis (percentage of phagocytosing haemocytes) was assessed over 6 fields of view per monolayer. Three monolayers were prepared for each treatment and the experiment was repeated twice. There was no significant difference between the 3 treatments (Kruskal-Wallis, $P = 0.38$, $n = 3$).

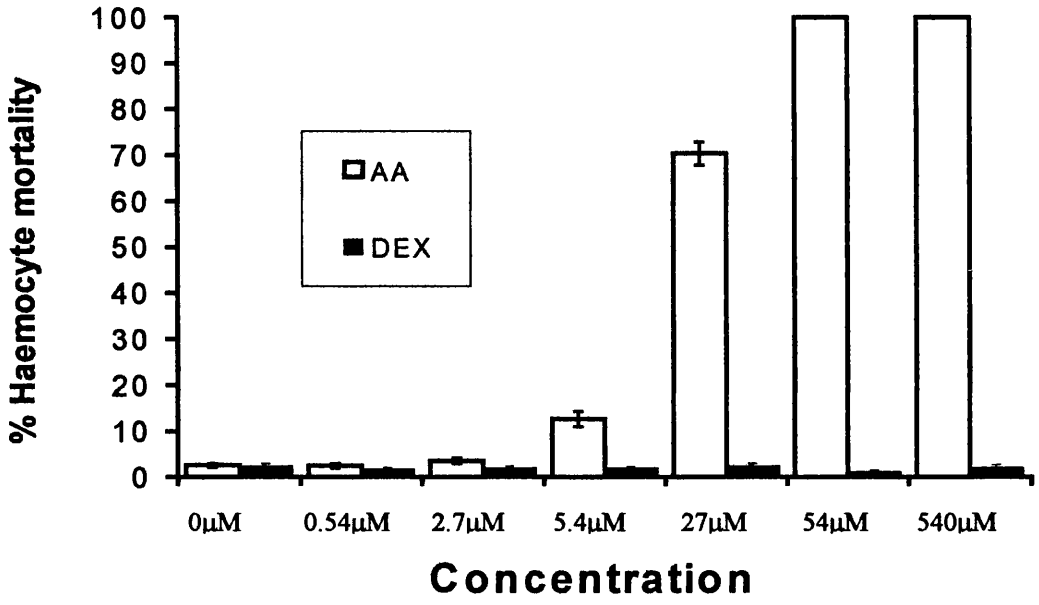


Fig. 8. Effect of different concentrations of dexamethasone (DEX) and arachidonic acid (AA) on haemocyte viability *in vitro*. Haemocyte monolayers were exposed to AA or DEX for 15 min and were then stained with trypan Blue to test for viability. The bars show percentage haemocyte mortality (mean ± SE) at the specified concentrations (n = 20).

6.3 Discussion

The idea that eicosanoids are important in insect immunity was first put forward by Stanley-Samuelson *et al.* (1991). It was shown that inhibition of eicosanoid biosynthesis reduced the clearance of the pathogenic bacterium *Serratia marcescens* from the haemolymph of *M. sexta* larvae and that this increased the associated mortality of the infected insects. In the same paper, it was shown that larvae of *M. sexta* contain all of the C-20 polyunsaturated fatty acids necessary for eicosanoid biosynthesis and are capable of converting AA into several eicosanoid metabolites. A later paper by this group (Miller *et al.*, 1994) showed that dexamethasone and other eicosanoid biosynthesis inhibitors inhibited nodule formation during artificial infections of *S. marcescens* in *M. sexta* and this effect was reversed by AA. This work provided a basis for what is now called the eicosanoid hypothesis (formalized by Stanley, 2000), which proposes that endogenous eicosanoids mediate nodulation reactions to bacterial challenge in insects.

Various authors have since built on this work, and there is now much evidence of the same general type to show that eicosanoids may mediate innate immune responses to bacteria in a wide range of insects. However, the question of whether eicosanoids also modulate defence reactions directed against fungi has so far not been addressed.

This study examines the effect of eicosanoid biosynthesis inhibitors on the immune responses of *M. sexta* larvae that had been challenged with the fungal entomopathogen *M. anisopliae*. The project had three main aims: (1) to investigate whether eicosanoid biosynthesis inhibitors could impair the ability of the insect to defend itself against a fungal pathogen, (2) to assess the importance of eicosanoid biosynthesis in nodule formation toward a fungal pathogen, (3) to investigate whether eicosanoid biosynthesis inhibitors have a direct effect on insect haemocytes responses to fungi.

M. anisopliae is an entomopathogenic fungus with a broad host range. I chose to use the isolate ME1 because it is highly pathogenic to *M. sexta* larvae (Samuels *et al.*, 1988). This fungus is pathogenic whether the conidia are applied topically (the normal route of natural infection) or given internally by injection into the haemocoel. In this study, I chose to inject *M. anisopliae* conidia because I wished to investigate the role of the insect's cellular immune response against the fungus. If conidia are applied topically,

then penetration of the host cuticle must be considered, independent of cellular immune processes. Moreover, since cuticle penetration takes some time, it would have been difficult to judge when to administer the pharmacological agents following inoculation. Thus, my experiments are concerned with the role of eicosanoids in the immune responses to fungi within the haemolymph, and do not address the question of whether these agents mediate defence responses to fungi during penetration of the insect's cuticle.

Injection of *M. sexta* with fungal conidia caused the insects to stop feeding and eventually the insects died. These symptoms were dependent on the dose of conidia injected, with higher doses causing a quicker reduction in weight gain and earlier mortality (i.e. increased speed of kill). Reduction of weight gain and earlier mortality were taken as measures of the success (growth) of the fungus within the insect.

The first aim was to determine whether eicosanoid biosynthesis inhibitors caused increased susceptibility of the insect to the fungus. The results were unequivocal. Infected larvae given dexamethasone died significantly sooner than those given the carrier (ethanol) alone. This effect was completely abolished by the co-addition of arachidonic acid, an eicosanoid precursor. Previous studies, using bacterial pathogens have reported similar findings, in which dexamethasone enhances the insect's susceptibility to the bacteria (Connick *et al.*, 2001; Park and Kim, 2000;Stanley-Samuelson *et al.*, 1991). I believe, however, that the present work is the first to show that dexamethasone also increases the susceptibility of an insect to an entomopathogenic fungus.

Weight gain of infected larvae was also reduced by dexamethasone and all the other eicosanoid biosynthesis inhibitors tested. Inhibition of weight gain gives a measure of the extent of mycosis, so these data suggests that the action of the inhibitors promotes a haemocoelic environment conducive to fungal growth. There was no effect on weight gain in larvae treated with any of the inhibitors alone (i.e. without fungus) suggesting the effect was due to enhanced fungal infection. Ideally, I would wish to test the effects of pure eicosanoids on the insect immune response to the fungus. Unfortunately, at present this is not possible because (a) the identities of the relevant endogenous eicosanoids are unknown, and (b) eicosanoids are unlikely to have a sufficiently long half-life *in vivo* to be useful in the kinds of experiments described in this chapter.

Our data nevertheless strongly suggest that eicosanoid/AA biosynthesis is important for the normal functioning of *M. sexta* immunity against *M. anisopliae.* This conclusion presumes there are no direct stimulatory effects of the drugs on fungal growth in the experiments. I tested the effects of dexamethasone and AA on the growth of *M. anisopliae in vitro.* I found that these compounds did not affect the ability of the conidia to germinate or of the emergent hyphae to grow at the same concentrations used in our *in vitro* microaggregation assays.

Our conclusion is also largely based on the effects of pharmacological agents that, although well characterised in mammals, are less so in insects. It cannot be certain that the effects of the drugs tested are due to specific inhibition of eicosanoid biosynthesis as they may have other, non-specific effects. However, the fact that addition of AA reverses the immunocompromising effects of dexamethasone, as would be expected if the action of this drug was to prevent AA synthesis, supports my interpretation. Further support comes from the following additional evidence. (1) Several of the other inhibitors tested have also been shown to inhibit eicosanoid-mediated processes in *M. sexta* and other insects (Stanley-Samuelson *et al.*, 1991; Stanley-Samuelson, 1994, Jurenka *et al.* 1999). (2) There is independent evidence that the eicosanoid biosynthetic pathway operates in *M. sexta.* The synthesis of various eicosanoids from labelled AA in *M. sexta* has been previously established in two immune related tissues, the haemocytes (Gadelhak *et al.*, 1995) and the fat body (Stanley-Samuelson and Ogg, 1994). Further, Jurenka *et al.* (1999) report that in the true armyworm *Psuedaletia unipuncta*, eicosanoid production is induced upon bacterial challenge and that this synthesis is inhibited by eicosanoid biosynthesis inhibitors (3) Both indomethacin and esculetin have been shown specifically to inhibit eicosanoid biosynthesis in *M. sexta* tissues (Gadelhak *et al.*, 1995; Stanley-Samuelson and Ogg, 1994).

The second aim of the study was to assess the significance of nodule formation during fungal infections and to study the effect of eicosanoid biosynthesis inhibitors on this process. Nodule formation is commonly said to be an immediate response to bacterial invaders (Horohov and Dunn, 1983; Lackie, 1988; Ratcliffe and Walters, 1983; Stanley *et al.*, 1999) and although studies have shown that fungi can elicit formation of nodules (Gunnarsson and Lackie, 1985; Hung *et al.*, 1993; Vey and Fargues, 1977), its

importance during fungal infections is not well established. My data reveals that injected *M. anisopliae* conidia provoke a strong nodule formation response in *M. sexta*. The number of mature nodules found in dissected larvae 24 h after injection of conidia was high (108 nodules). This is comparable to the levels of nodulation reported by Miller *et al.* (1994) who injected *M. sexta* with the bacterial pathogen *S. marcescens* (148 nodules after 24 h).

When dexamethasone was co-injected with the conidia, the number of nodules was strongly reduced compared with fungus-injected controls, and this effect was reversed by AA. These *in vivo* nodulation results support similar findings with bacteria (Jurenka *et al.*, 1997; Stanley-Samuelson *et al.*, 1997), bacterial LPS (Bedick *et al.*, 2000), latex microspheres (Mandato *et al.*, 1997), and most recently, encapsulation reactions to parasitoid eggs (Carton *et al.*, 2002). The eicosanoid hypothesis can therefore be extended to fungal infections as well as bacteria; I suggest that eicosanoids may play an integral role in the formation of nodules in response to all foreign elicitors.

Our results also demonstrate the importance to the insect of the nodulation reaction in defence against a fungal pathogen. Larvae in which nodulation was suppressed were more susceptible to mycosis (revealed by the mortality and weight gain data). Therefore, it may be concluded that under normal conditions, nodule formation plays a significant role in limiting and slowing the progress of mycosis, as is also the case for bacterial infections.

Our final aim was to determine whether eicosanoid biosynthesis inhibitors suppress nodule formation by acting directly or indirectly on *M. sexta* haemocytes. Nodule formation is a complex process involving several distinct cellular processes (Ratcliffe and Rowley, 1979), including recognition of a foreign elicitor; adherence to the elicitor; aggregation of haemocytes (of more than one type); haemocyte degranulation; haemocyte movement and shape change; the possible secretion of substances toxic to microbial invaders; melanization; and haemocyte death. It is difficult to replicate the whole sequence of nodule formation *in vitro*. In this work, an *in vitro* assay was used as a surrogate for nodule formation, in which washed haemocytes are exposed to fungal conidia and react to them by the formation of microaggregates of 5-20 cells. This assay detects at least the first three of the above components of the nodule formation

response. Miller and Stanley (2001) have recently published a similar, but not identical *in vitro* microaggregation assay using *M. sexta* haemocytes exposed to the bacterium *S. marcescens*.

Although there was a low background level of microaggregation in non-challenged *M. sexta* haemocytes, the number of microaggregates formed was significantly higher when haemocytes were challenged with fungal conidia. Because dexamethasone significantly suppressed the number of microaggregates formed, I conclude that the effect of this inhibitor on fungally-elicited haemocyte microaggregation is direct. Miller and Stanley (2001) also showed that dexamethasone could inhibit bacterially-elicited microaggregation *in vitro*, and similarly concluded that the action of dexamethasone on haemocytes was direct.

Although in my experiments the co-addition of low concentrations of AA reversed the effect of dexamethasone to some extent, the increase in the number of microaggregates was not statistically significant. In similar experiments, Miller and Stanley (2001) were able to show a complete and significant reversal of the effects of dexamethasone with AA. There are several possible reasons for this difference in the results including (i) differences in the concentration of AA used in the assay (4.3 μM, compared to the 2.7 μM used here) (ii) a different microbial elicitor was used in our assay (Miller and Stanley used the bacterium *Serratia marcescens*) (iii) the haemocytes used in our assay had been washed in calcium free saline during preparation. While this treatment reduced coagulation reactions, it is possible that it also rendered the haemocytes less responsive to AA.

Interestingly, AA appears to be considerably more toxic to *M. sexta* haemocytes *in vitro* than to those of the greater wax moth, *Galleria mellonella*. Mandato *et al.* (1997) reported that when *G. mellonella* haemocytes were exposed to 100 μM AA *in vitro*, they did not suffer a significant loss in viability. In the present study, *M. sexta* haemocytes were much more sensitive to AA as revealed by the toxicity assay (Fig. 8). *M. sexta* haemocyte viability was significantly affected at concentrations 5.4 μM and above, considerably less than the concentration used by Mandato *et al.* (1997).

The study by Mandato *et al.* (1997) also revealed that dexamethasone suppressed phagocytosis by *G. mellonella* haemocytes *in vitro*. This was tested in the present study and was found not to be the case for *M. sexta* haemocytes at similar *in vitro* concentrations (Table 1). However, it is noted that Mandato *et al.* (1997) incubated *G. mellonella* haemocytes with carboxylated latex beads to elicit phagocytosis while in the present study, bacteria were used. This is important as the recognition and signal transduction of different foreign stimuli by haemocytes may depend on eicosanoid pathways to different degrees. It is also likely that *M. sexta* haemocytes are not as responsive to dexamethasone as *G. mellonella* haemocytes and thus their ability to phagocytose may not have been impaired at the concentration of dexamethasone used.

Nodule formation by insects as a response to bacterial infection is now well established in the literature (e.g. Horohov and Dunn, 1983; Ratcliffe and Rowley, 1979; Ratcliffe and Walters, 1983). The present work has established that nodule formation is also an important response to fungal entomopathogens and that this response may be modulated by eicosanoids. Our demonstration that suppression of nodule formation is correlated with an increase in fungal virulence shows that the nodulation response is an important factor in defending the insect against fungal invasion.

Exactly how the eicosanoids are involved in the insect immune system is unknown and would be difficult to determine. Nodulation is a complex process (as described above) and eicosanoids could potentially play a role at any stage of this response. Eicosanoids are a diverse family of compounds that exhibit (at least in mammals) a multitude of cellular and physiological activities (for a review see Rowley *et al.*, 1998). They have been reported to be important in cell-cell signalling (Marcus *et al.*, 1987; Peters-Golden, 2000), cell adhesion (Filep *et al.*, 2000; To and Schrieber, 1990), cell aggregation (Ford-Hutchinson *et al.*, 1980), chemotactic responses (Arnould *et al.*, 2001; Bowers, 2000; Monneret *et al.*, 2001), apoptosis (Fiore, 2000; Grelli *et al.*, 1995), cytokine gene expression (Alaya *et al.*, 1994) nitric oxide synthase induction (Amin *et al.*, 1995), reactive oxygen species generation (Serhan *et al.*, 1982) gene regulation and protein synthesis (Yao *et al.*, 1999). All of these cellular activities may be important in nodule formation and thus the potential for eicosanoids to be involved in this process is high.

In order to become established in its host, a successful entomopathogen must escape the vigilance of the insect immune system during the early stages of infection. Given the importance of eicosanoid signalling to the insect immune system, interference with eicosanoid metabolism would seem to be a sensible strategy, and thus a potentially important virulence factor for an entomopathogen. I predict that at least some entomopathogens will be found to inhibit eicosanoid signalling in their insect host.

Chapter 7

General Discussion

The present study aimed at gaining an insight into the mechanisms of cellular immunity in the tobacco hornworm *Manduca sexta*. Cellular immune responses towards invading microorganisms (phagocytosis and nodule formation) are the most important lines of defence in insects after the cuticle has been breached and this is represented by the high level of research in this area. Although the underlying molecular mechanisms of phagocytosis and nodule formation are still relatively unknown, these processes have been well characterised and research over the last decade has started to identify important factors that are involved. *Drosophila melanogaster* is proving a useful genetic tool in dissecting cellular immune responses (De Gregorio *et al.*, 2001). However, the small size of *Drosophila* makes immunological assays difficult and the immune system of this insect appears to be uncharacteristic of other insect models studied (Rizki and Rizki, 1984a). *M. sexta* is a well known insect of large size that can be easily dissected and provides large quantities of haemolymph for *in vitro* immunological assays.

HP Cells are important in *M. sexta* immune response

The haemocytes of *M. sexta* have been characterised previously into five types, namely granular cells (GR), plasmatocytes (PL), spherulocytes (SP), oenocytoids (OE) and prohaemocytes (PR) (Geng and Dunn, 1989; Horohov and Dunn, 1982; Willot *et al.*, 1994). The hyperphagocytic (HP) cells described in the present study have not been reported before. Although these cells are clearly different to the other haemocyte types on the basis of function and morphology when spread, it is possible that that have been overlooked in the past because (a) their low frequency in the haemolymph (b) their thinness when spread on glass (c) they have been considered as plasmatocytes because of their spreading ability. Using only functional criteria to classify insect haemocytes is inherently flawed due to the variation in function of morphologically similar haemocyte types between different species. For example, PL are the main phagocytic cell type in *Galleria mellonella* (Anggraeni and Ratcliffe, 1991) whilst in *Bombyx mori* only GR function as phagocytes (Wago, 1991). In the present study, HP cells were distinguished

from other haemocyte types based on both functional and morphological criteria and thus the possibility that they constitute a separate haemocyte class cannot be discounted.

HP cells offer the insect an efficient means of immune surveillance and removal of bacteria from the haemolymph. Previously, nodule formation has been considered to be of greater importance than phagocytosis for the removal of bacteria from the haemolymph of *M. sexta* larvae (Horohov and Dunn, 1983). Our results, however, suggest that in this insect phagocytosis by HP cells is responsible for the majority of bacteria removed from the haemolymph following an injection of high doses of bacteria. Although the involvement of a class of professional phagocytes (i.e. HP cells) seems a logical immune strategy, they have not been reported previously in insects. However, given that insect immunity resembles the innate immune system of vertebrates, it would not be surprising to find truly 'professional' phagocytic cell types in insects.

I have shown in this study that HP cells intimately associated with microaggregates of haemocytes in immune-challenged larvae. This suggests an alternative to the well-established model for nodule formation proposed previously by Rowley and Ratcliffe, 1981. Nodule formation has been considered to be a biphasic process with the first phase involving entrapment of bacteria by granular cells and the second phase involving the flattening of plasmatocytes around the nodule core to form a multicellular sheath. In the present study, GR were indeed involved in the early phase of nodule formation but, importantly they were found associated with HP cells in the early microaggregates. My model of nodule formation, however, differs from that of Rowley and Ratcliffe (1981) and is consistent with the hypothetical model of Davies and Siva-Jothy (1991) that a recognition haemocyte type (i.e. the HP cell) 'patrols' the haemolymph for the presence of nonself. HP cells were found to be very good at recognising and binding *E. coli,* to the extent that most bacteria bound to HP cells and few to other haemocyte types. This was found to be the case *in vitro* and during the *in vivo* phagocytosis assays where the majority of phagocytosed *E. coli* were within HP cells. After recognition, binding and hyperphagocytosis of the bacteria, HP cells appear to act as nuclei for other haemocytes in nodule formation. Although phagocytosis has been previously shown to occur concomitantly with nodule formation (Horohov and Dunn, 1983; Ratcliffe and Gagen, 1977), the level of phagocytosis exhibited by the haemocytes was limited and the involvement of cells fitting the description of HP cells was not reported.

The involvement of lectins is an important issue in the proposed model. Lectins are thought to act as bridging molecules between the bacteria and the haemocyte surface and have been shown to enhance phagocytosis (Jamori and Natori, 1992; Pendland *et al.*, 1988; Wilson *et al.*, 1999). Although in my experiments the haemocytes and bacteria were washed free of plasma components prior to being presented with the bacteria *in vitro*, the secretion of opsonins such as lectins into the supernatant could have occured. *M. sexta* has been shown to possess a lectin called scolexin that was found concentrated in nodules with bacteria and coagulum (Kyriakides *et al.*, 1993). Thus, *in vivo*, lectins are likely to play an important role in phagocytosis by HP cells and probably enhance the hyperphagocytic process. A useful experiment would be to assess the difference in response of HP cells to opsonised and unopsonised bacteria.

HP cells were found to phagocytose massive numbers of bacteria. To our knowledge this is the highest level of phagocytosis reported for any animal cell. Confocal microscopy revealed that HP cells were completely full of bacteria. Most studies with macrophages, including those in which the bacteria had been pre-opsonised, show less than 20 ingested bacteria (Drevets and Campbell, 1991; Oda and Maeda, 1986). In comparison HP cells had over 350 bacteria per cell. Without the use of the confocal microscope slices it would have been extremely difficult to count the very large numbers of tightly packed bacteria in HP cells.

This work has various implications: (1) HP cells provide a useful tool to study phagocytosis as these specialist cell types must possess large amounts of the components of the phagocytic apparatus (including an abundance of receptors and specialised signal transduction pathways). (2) HP cells may be present in other insects and are likely to be extremely important in insect immunity (3) HP cells would be a likely target for specialist entomopathogens as they appear to be the most important haemocyte type, at least in *M. sexta*.

P. luminescens suppresses cell mediated immune responses

The influence of *P. luminescens* on the phagocytic activity of *M. sexta* haemocytes was assessed. At the time of this study, HP cells had not been identified and thus were not be considered in the context of immunosuppression by *P. luminescens*. This bacterium is

highly virulent towards *M. sexta* and, like other entomopathogens, must possess mechanisms to avoid or suppress the insect immune response. A common theme among the pathogenicity strategies of entomopathogens is the suppression of nodule formation and phagocytosis (Boucias *et al.*, 1995; Huxham *et al.*, 1989; Richards and Edwards, 2000). This may be through the secretion of immuno-toxins. However, some entomopathogens are known to change their surface properties and therefore it is possible they are not recognised by the insect. *Beauveria bassiana* has been hypothesised to employ this strategy *in vivo.* This fungus sheds its cell wall and is apparently not recognised by immunocompetent haemocytes, possibly due to the adsorption of host derived haemolymph proteins (Pendland *et al.*, 1993). *B. bassiana* also produces immunosuppressive factors (reviewed by Boucias *et al.*, 1995), thus as might be anticipated there are a variety of strategies to overcome or avoid the insect immune system

In the present study *P. luminescens* strain W14 induced both nodule formation and phagocytosis but only when the bacteria were heat-killed. Live *P. luminescens* provoked little response. The use of heat to kill the bacteria has the disadvantage of possibly causing alterations to the bacterial cell surface. *P. luminescens* did however produces metabolites *in vitro* that suppress both phagocytosis and nodule formation suggesting the bacterium can actively suppress haemocyte function rather than avoid recognition. *P. luminescens* has been shown to produce a variety of different metabolites *in vitro* including toxin complexes (Tc) (Bowen *et al.*, 1998), proteases (Bowen *et al.*, 2000; Schmidt *et al.*, 1988), lipases (Clarke and Dowds, 1995) and lipopolysaccharide (Dunphy, 1995), all of which are potentially immunosuppressive. Although the identity of the anti-phagocytic factor is unknown, this factor is either produced in high quantity in the supernatant or is very potent towards the haemocytes as a 1 in 100 dilution of the supernatant had significant anti-phagocytic activity.

The production of toxin complexes (Tc) *in vivo*, which destroys the insect's midgut and lead to insect death, is one component of *P. luminescens* pathogenesis (Blackburn *et al.*, 1998). The present study suggests that in order to persist long enough to multiply and deliver such toxins *in vivo, P. luminescens* suppresses the cellular immune responses of phagocytosis and nodule formation.

Eicosanoids and nodule formation

A wide variety of factors have been identified that modulate haemocyte responses. These include, hormones (Baines et al., 1992; Goldsworthy *et al.*, 2002; Gateff, 1998; cited by Vilmos and Kurucz, 1998), haemocyte adhesion molecules such as peptides containing an RGD sequence that affect encapsulation (Pech and Strand, 1995); proteins that inhibit haemocyte aggregation such as lipophorin (Coodin and Caveney, 1992) and the haemocyte aggregation inhibitor protein (Kanost *et al.*, 1994); biogenic amines and octopamine that enhance phagocytosis and nodule formation (Baines *et al.*, 1992); peptides containing the sequence ENF that affect haemocyte spreading and adhesion (Strand *et al.*, 2000); phagocytosis stimulating factors (Wiesner *et al.*, 1996); and the eicosanoids which have been shown to be important in nodule formation during a bacterial infection (Miller *et al.*, 1994).

In the present study it was shown that nodule formation in *M. sexta* makes a significant contribution to host defence during a fungal infection and that this immune response toward fungi is influenced by eicosanoid biosynthesis inhibitors. The finding that eicosanoid biosynthesis inhibitors suppress nodule formation and enhance mycosis suggests that the eicosanoids are important in the nodule response toward fungi and imply nodule formation is important at slowing the growth of the fungus *in vivo*. It is important to note that intrahaemocoelic injection of conidia is not the natural route of infection for the fungus and the immune response to blastospores (during a natural infection) may be quite different. The reasons for injecting the conidia were outlined in the discussion of Chapter 6. However, crucially, larvae dissected after a topical infection with *M. anisopliae* had many nodules lining the body cavity (results not shown) suggesting nodule formation is a natural response to this fungus during infection.

It seems likely that eicosanoids modulate both nodule formation and encapsulation (i.e. haemocytic aggregation) to all foreign agents. We discussed in chapter 6 the possible roles that eicosanoids may play in nodule formation. Eicosanoid biosynthesis inhibitors have now been shown to inhibit nodule formation toward fungi (Dean *et al.*, 2002), bacteria (Miller *et al.*, 1994), LPS (Bedick *et al.*, 2000) and latex microspheres (Mandato *et al*, 1997) and recently have been shown to suppress the encapsulation response to a parasitoid wasp (Carton *et al.*, 2002). Although the actions and selectivity of the

inhibitors that were used in previous and the present study have been characterised in mammals, they have not been well characterised in insects. Thus, the possibility of non-specific effects of these inhibitors cannot be discounted. The fact that the suppressive effect of dexamethasone on nodule formation in the present study was reversed in the presence of AA is support for our hypothesis that eicosanoids are specifically involved in nodule formation. In addition, endogenous eicosanoids and the biosynthetic apparatus that synthesise them have been identified in insect tissues and their biosynthesis was specifically inhibited by eicosanoid biosynthesis inhibitors (Jurenka *et al.*, 1999; Stanley-Samuelson and Ogg, 1994; Tunaz *et al.*, 2001). Thus, the use of these inhibitors for assessing the role of eicosanoids in insect immunity seems reasonable.

Haemocytes spread extensively in response to infection

All of the cellular immune responses in insects (phagocytosis, nodule formation and encapsulation) require haemocytes to change shape and spread around the foreign objects. The outcome depends on the size of the foreign particle, as small particles are phagocytosed or induce nodule formation while larger objects are encapsulated. In the present study, the response of the haemocytes to injection with pathogenic or non-pathogenic bacteria, or infection with pathogenic fungi, was to spread dramatically when allowed to attach to a glass slide. Hyperspreading haemocytes or very large blood cells (VLBC) were induced by injection of laminarin (a source of the fungal cell wall polymer ß-1,3-glucan) into test larvae. We are unaware of any previous reports of this type of immune response toward infection. During the later stages of mycosis, a dramatic reduction in haemocyte spreading occurred as VLBC were not seen in any late-stage infected larvae. This is consistent with the work of Hung *et al.* (1993) for *Beauveria bassiana* infection in *Spodoptera exigua* and similar results have been shown with other entomopathogenic fungi (Vilcinskas *et al.*, 1997) and parasitoids (Strand and Noda, 1991).

Whether VLBC are new cell types or primed (differentiated) plasmatocytes with increased spreading ability, it is clear that the observed spreading of the haemocytes is an immune response of the insect and not a pathological effect of infection. This was shown as VLBC appeared with chemical stimulation (laminarin injection) or injection with other organisms. The response appears to be integral to nodule formation as VLBC were

invariably associated with microaggregates. Haemocyte spreading has been reported as a common phase of nodule formation. It facilitates for the formation of flat, multicellular layers around the nodule and enhances the entrapment of invaders (Rowley and Ratcliffe, 1981). It is also possible that the spreading effect may be a consequence of an increased phagocytic potential of the cells. As mentioned in the HP cell chapter, spreading by blood cells on a glass slide has been considered a 'frustrated phagocytosis' in that the cell is attempting to engulf the slide (Swanson and Baer, 1995). Although VLBC were relatively non-phagocytic (as they did not readily phagocytose FITC-labelled *E. coli in vitro,* results not shown) compared with conventional plasmatocytes, this may have been due to their extreme spread morphology on the slide and in their native, rounded state, it is possible that they may be adept phagocytes. This requires further work.

The spreading response to infection described in this study also needs further investigation. Important questions that remain unanswered include: what happens to the VLBC count during a non-pathogenic bacterial or fungal infection? Why do the VLBC disappear as a fungal infection progresses? What are the signals for the induction of VLBC? Why the there two different VLBC morphologies? What are the origins of VLBC? Radiolabelled thymidine could be used to identify the time of production and life span of the VLBC as well as revealing whether they are new or pre-existing cell types. Work with the *Drosophila* lymph gland has shown that this organ responds to wasp parasitisation through increased cell division and differentiation (Sorrentino *et al.*, 2002) and similar techniques with the *Manduca* haemopoietic organ would allow the determination of the importance of this organ in the VLBC response.

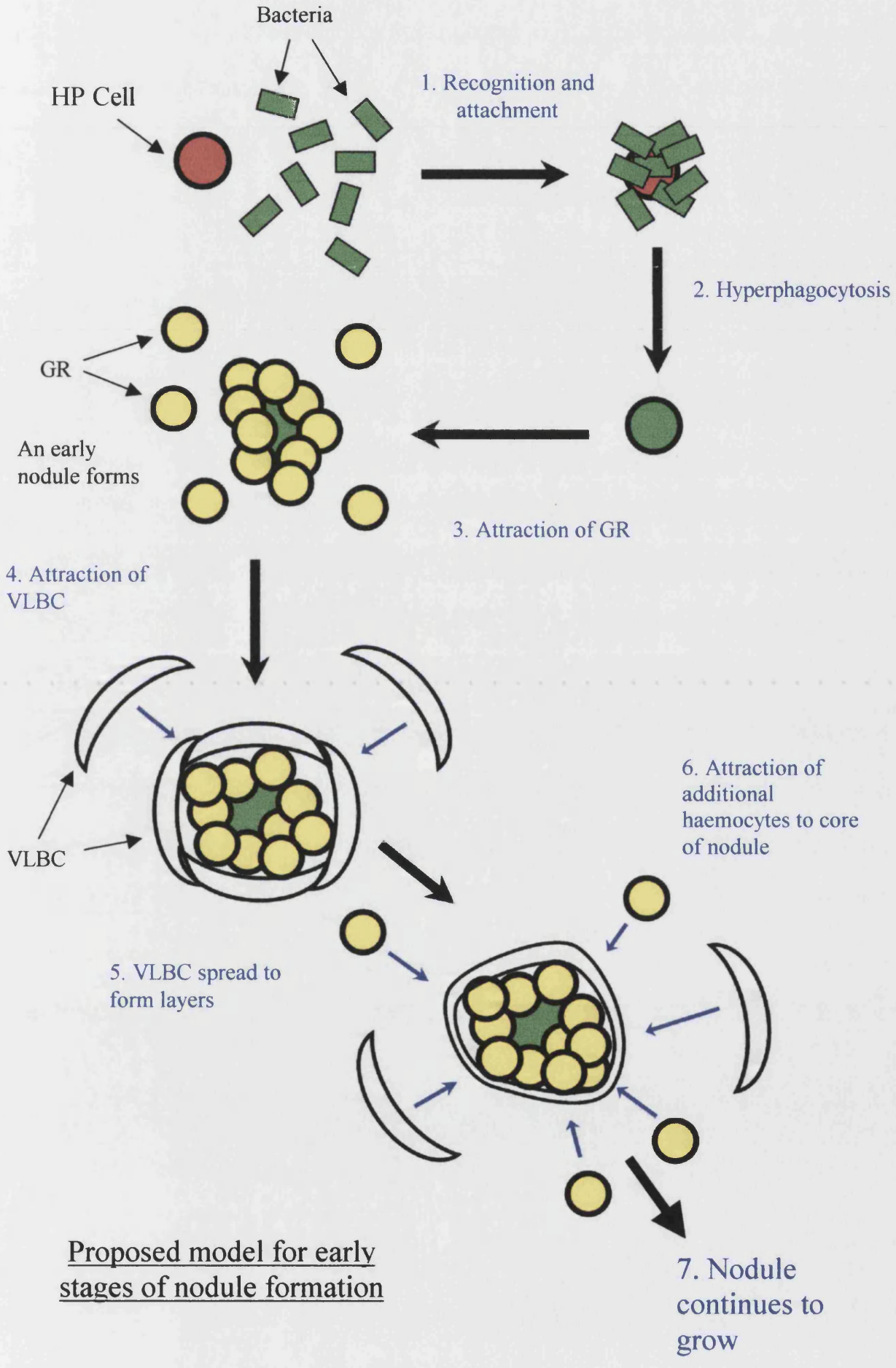
It was not possible to induce haemocytes in healthy monolayers to spread and display morphologies of the VLBC. This suggests that (a) VLBC are indeed new cell types released into the haemolymph upon infection or (b) the signalling factor that induced VLBC from normal haemocytes was not present in these induction experiments. Although infected plasma was tested at different stages of infection, the many reactions that occur in the haemolymph upon bleeding, or the possible instability of the factor *in vitro,* may have been sufficient for the activity to be lost. It was found, however, that spreading was inhibited in the presence of late-stage infected plasma and *B. bassiana* 304 culture filtrate suggesting the action of metabolites from this fungus were responsible for the loss in VLBC occurrence during late-stage infection.

Proposed model for nodule formation in *M. sexta*

The relationship between the spread haemocytes that are induced upon infection (VLBC) and the HP cells is an important issue. These two cell types are different in terms of morphology and function. HP cells are always present in the haemolymph whereas VLBC appear only upon immune challenge. However, both cell types spread extensively. Both label with an antibody that labels conventional plasmatocytes and both appear to be involved in nodule formation.

I propose the following model for the early stages in nodule formation in *M. sexta* based on the findings with HP cells and VLBC. The model is given as a diagram overleaf and can be summarised by:

(1) Foreign particles in the haemolymph are quickly recognised by and bind to HP cells. Infection stimulates the induction of haemocytes with extreme spreading ability (VLBC) within the haemolymph (2) Phagocytosis of the bacteria by HP cells causes a change in the interaction between the HP cells and other haemocyte types resulting in haemocyte aggregation. GR are attracted to the HP cell and adhere to it, forming a microaggregate or early nodule. This may occur through a change in the HP cell surface that enhances cell-cell adhesion, or through the secretion of factors such as chemoattractants. GR within the nodule often degranulate and this may trap additional bacteria and act in attracting haemocytes (3) Microaggregates attract VLBC, which attaches to and spreads around the cells of the microaggregate. The VLBC forms flat layers around the cells, essentially isolating the nodule core, the entrapped bacteria and the HP cell (5) additional haemocytes (GR, PL or VLBC) attach to the nodule and when the nodule reaches a large enough size it settles out of circulation and attaches to the body wall (6) many of the haemocytes within the nodule die, possibly due to apoptosis or toxic metabolites released within the nodule. High levels of haemocyte death in nodules were observed in the present study (7) during the process of nodule formation the bacteria may be killed by toxic metabolites from the HP cell or surrounding haemocytes or from nutrient/oxygen depletion (8) the involvement of eicosanoids may occur at many of the stages described i.e. cell-cell signalling; cell adhesion; chemoattraction; stimulation of toxic metabolites; induction haemocyte apoptosis; regulation of phagocytosis by HP cells; signal transduction and gene transcription during nodule formation.



Proposed model for early stages of nodule formation

Concluding remarks

This thesis furthers our knowledge of cellular immunity in *M. sexta* and has wider implications for insect immunity in general. The 'eicosanoid hypothesis' (formalized by Stanley, 2000) that eicosanoids mediate nodule formation to bacterial infections, can now be extended to fungi. I have shown that suppression of nodule formation enhances mycosis, revealing the importance of this response during fungal invasion. It is predicted that hyperphagocytic cells and the spreading response of haemocytes will prove to be an important component of the immune system of many other insect species. The HP cells of *M. sexta* are clearly different from the other haemocytes in terms of morphology (when spread) and function and the evidence presented here reveals their importance to the immune system. Taken together, eicosanoids, HP cells and VLBC all appear to be involved in nodule formation in *M. sexta* and this demonstrates the complexity of the nodule response. An interesting issue that was not addressed in the present study is the regulation of HP cells and VLBC, in particular the possible involvement of eicosanoids. Finally, *P. luminescens* was shown to produce factors that suppress phagocytosis and nodule formation. Although this is important from a pathogenesis viewpoint, the characterisation of these factors and their modes of action could provide important information about the mechanisms of cellular immune responses.

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